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Domain-specific expression of PIN1 polar auxin transporter in Arabidopsis thaliana

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ABSTRACT

Ma, Guojie. M.S., Purdue University, May 2016. Domain-Specific Expression of *PIN1* Polar Auxin Transporter in *Arabidopsis Thaliana*. Major Professor: Angus Murphy.

Auxin as one of the most important natural hormones regulates many aspects of plant growth and development, including cell division, expansion and elongation, plant patterning and tropic responses. Works over the last decades have uncovered that plasma membrane asymmetric localized PIN-FORMED (PIN) auxin efflux carriers are critical for polar auxin transport and local concentration gradients. The five canonical PIN (PIN1, 2, 3, 4, 7) members exhibit overlapping roles in plant root development. Among them, only PIN1 is displaying some unique functions in some specific layers in auxin mediated shoot organogenesis and phyllotactic patterning. Such as in the L1 layer, PIN1-mediated auxin accumulation is required for organ initiation and arrangement. Reversible phosphorylation, cell wall, plasma membrane composition and subcellular trafficking offered flexible regulation of PIN1 polarity in response to a variety of stimulations and subsequent direction of auxin fluxes, which is important for plant patterning, organogenesis and tropism.

PREFACE

This thesis mainly focused on dissecting PIN1 function in specific domains in *Arabidopsis*. The detailed analysis of PIN1-mediated auxin fluxes functioning in different layers will provide new evidence for PIN1 important function. PIN1 was initially identified for its prominent pin-like inflorescence head. Although slight deformation of vascular has been observed in *pin1* mutants, pharmacological and florescence tag fusion analysis has shown localized PIN1-mediated auxin polar transport primarily regulates shoot organogenesis. However, all the conclusions were made from its spatial and temporal expression pattern.

In order to find in which domains(s) PIN1 function is essential for shoot organogenesis, we determined to express *PIN1-GFP* driven by domain-specific promoters. Seven domain-specific promoters *ATML1, ATHB-8, CUC3, CLV3, WER, FIL, SCR* have been selected according to their domain specificity and PIN1 expression pattern. Three domain-specific promoter transgenic lines with *pin1-7* heterozygous background and proper domain-specific expression were selected to analyze PIN1-mediated auxin transport functioning in different domains in *Arabidopsis*. Previous studies have found *pin1* mutants showed 20% reduction in polar auxin transport in the whole plant and 90% reduction of IAA transport in the first 2.5 cm of inflorescence. This indicated that PIN1 is more important for localized auxin fluxes rather than long-distance

transport. Although researches also found reduced root paical meristem size, slightly inhibited primary root elongation in *pin1* mutants, the most pronounced defective phenotypes were observed in the shoot, including organ separation, leaf venation, leaf serration, floral meristem formation as well as primordia formation and development. This means PIN1 is playing critical roles in shoot organogenesis. The current test results are described in chapter one.

Then, in order to find at which stage PIN1 function is essential for shoot organogenesis, we determined to estradiol-inducible system, which is developed by Ari pekka lab which have been used in chapter two. Using this system, *PIN1* will be turned on at the seedling stage and in the middle of vegetative growth stage and reproductive stage to analyze how *pin1* mutants grow. Using this system, we successfully controlled *PIN1* expression at different domains, however due to the diffusibility, leakiness, and toxicity for long-term application, we did not fulfill the objectives.

In chapter one, we are mainly focusing on analyzing *pin1* phenotypes with each promoter driven expression *PIN1-mGFP* and comparing them with wild type and *pin1* mutant. From the confocal imaging of the transgenic lines, we found some of the tissue-layer specific promoters were not as specific as people reported. For example, the CUC3 a member of the CUP-SHAPED COTYLEDON (CUC) family of transcription factors were identified expressing at the boundary zone-a narrow region between SAM and the peripheral zone, but we observed weak *PIN1-mGFP* expression in the vasculature under the *proCUC3* driven. With different promoter control, we also observed the level of *PIN1-GFP* intensity were distinct. This observation needs to be confirmed by qRT-PCR.

Phenotypic analysis found that *PIN1* expression in the boundary zone driven by *proCUC3* and CZ controlled by *proCLV3* was not sufficient to complement *pin1* defective phenotypes. Ecotopic expression of *PIN1* driven by *proSCR* would cause prominent phenotypes, which were observed in *pin1* homozygotes.

This project was designed to better understand PIN1 function and domains are crucial for PIN1 associated shoot organogenesis. Understanding of mechanisms of plant growth is especially critical for agriculture production and natural resources from plant. All these plant-based materials are derivatives of the SAM. Results from this project could provide theoretical base for future genetic modification to improve plant growth.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Auxin is a ubiquitous signaling intermediate in plant growth and development. Naturally-occurring active auxins include the predominant signaling molecules, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), phenylacetic acid (PAA), and, in some species, 4-chloroindole-3-acetic acid (4-Cl-IAA). Synthetic auxins used in agriculture include 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), 3,6-dichloro-2-methoxybenzoic acid (dicamba), and 4-amino-3, 5, 6-trichloropicolinic acid (picloram) (reviewed by Korasick et al., 2013; Zhao, 2013). At young developmental stage, auxin is primarily derived from the shoot apex, rapidly dividing tissues and primordia (Ljung et al., 2001, 2005, 2013). The root also has been reported as an important auxin source (Ljung et al., 2005). In the stem, auxin is basipetally transported to the action sites: the root auxin is moved acropetally to the root tip and refluxed from the root apex to the upper zones through epidermal and cortical tissue layers (Zažímalová et al., 2010; Peer et al., 2011). This defines the polar auxin transport (PAT). The aerially derived auxin has been reported to be critical for lateral root primordia (LRP) emergence in the very early stage seedlings (Ljung et al., 2001, 2005). However, increasing evidences have demonstrated that both long-distance auxin transport and localized auxin

biosynthesis and transport are essential for every aspect of plant development (Ljung et al., 2001, 2005; Cheng et al., 2006, 2007; Robert et al., 2013, 2015).

1.2 Cellular Auxin Transport

As a weak organic acid, IAA exists as an associated form (IAAH) in the apoplast-acidic environment, which can enter into the cell freely by diffusion and the process is also accompanied by active influx carriers AUXIN RESISTANT (AUX1) and LIKE AUX1 (LAX) proteins (Peer et al., 2011; Swarup, 2012). In the phloem and xylem, auxin is translocated along with fluids in a high velocity. Auxin is dissociated into an ionic form (IAA^-) in the slightly alkaline cytosol and trapped inside the cell by the plasma membrane. The highly regulated specific PM positioned auxin efflux and influx carriers are necessary to facilitate auxin transported polarly through cell-to-cell manner (Blakeslee et al., 2005; Wiśniewska et al., 2006; Petrášek et al., 2009; Křeček et al., 2009; Peer et al., 2011). Findings from *Arabidopsis* indicate that ATP-BINDING CASSETTE subfamily B (ABCB) of proteins and PIN-FORMED (PIN) proteins are functioning as auxin efflux carriers in the PM (Reviewed by Křeček et al., 2009; Peer et al., 2011; Balzan et al, 2014). ABCB1/19 primarily mediates long-distance auxin transport from shoot to root alongside with polar-localized PIN proteins finely tune the directionality of localized auxin streams, which is important for many aspects of plant development (Noh et al., 2001; Geisler et al., 2005; Wiśniewska et al., 2006; Blakeslee et al., 2007). The single mutants of *b1* and *b19* exhibit very mild growth defects and mutation of *PINs*, especially *PIN1* cause pronounced organ defects (Reviewed by Křeček et al., 2009; Peer et al., 2011; Friml et al., 2015).

1.3 AUX/LAX Influx Carriers

Forward genetics has identified *AUX/LAX* gene family, which has been characterized as auxin influx carriers (Swarup et al., 2008; Yang et al., 2009; Vandenbussche et al., 2010; Swarup et al., 2012). In *Arabidopsis* genome, AUX/LAX protein family has four members AUX1, LAX1, LAX2, LAX3 and belongs to amino acid/auxin permease super family with multi-transmembrane spans (Swarup et al., 2008; Yang et al., 2009; Vandenbussche et al., 2010; Swarup et al., 2012). In vitro experiment using yeast system has proved that AUX1 and LAX3 can directly transport auxin with high-affinity (Yang et al., 2009). AUX1 mediated auxin transport has been reported essential for plant gravitropism and lateral root initiation (Swarup et al., 2008). *LAX3* expression has been observed highly correlated with lateral root primordia emergence (Péret et al., 2013). AUX1, LAX1 and LAX2 uptake activities appear necessary for the shoot and root pole formation during embryogenesis (Robert et al., 2015). Findings indicate that AUX/LAX proteins are cycled between PM and endosomal compartments through a pathway distinct from PIN1 (Klein-Vehn et al., 2006). The mechanism related to AUX/LAX trafficking is still not clear.

1.4 Efflux Carriers

In Arabidopsis genome, 21 ABCB transporters have been identified, however only ABCB1 and 19 have been characterized with high substrate specificity for auxin transport (Titapiwatanakun et al. 2009; Yang and Murphy 2009). Both have been observed localized on the PM with nonpolar manner (Noh et al., 2001; Geisler et al., 2005; Blakeslee et al., 2007). Mutation of B19 resulted in pleiotropic phenotypes including

epinastic cotyledons, loss of apical dominance, wrinkled rosette leaf margin and wavy primary root (Noh et al., 2001; Blakeslee et al., 2007; Mravec et al., 2008). Findings from *Arabidopsis* indicate that ABCB19 primarily mediates long-distance rootward auxin transport alongside with ABCB1 function in moving auxin out of the shoot (Noh et al., 2001; Geisler et al., 2005; Blakeslee et al., 2007). B19 also has been proposed inactivated by phosphorylation and created the auxin pool above the cotyledon nod for auxin redirection at the beginning of phototropic response (Christie et al., 2011). Polar-localized plasma membrane PIN proteins appear to be crucial for the direction of auxin stream and amplify the polar auxin gradients (Wiśniewska et al., 2006). Polar auxin transport created maxima and gradients are essential for establishment of apical-basal axis, regulation of plant patterning and responses to different stimuli (Petersson et al., 2009; Normanly, 2010; Křeček et al., 2009; Zažímalová et al., 2010; Peer et al., 2011; Robert et al., 2015).

PIN family genes, which are named after pin-like inflorescence head of the *pin*1 mutants, also display auxin efflux functions (Křeček et al. 2009; Petrášek and Friml, 2009; Peer et al., 2011). Eight *PIN* genes have been identified in Arabidopsis genome and extensively characterized regarding their expression, localization and developmental roles. PIN family is divided to two subgroups according to the size of the central hydrophilic loop, long PINs (PIN1-4, PIN7)- also called canonical PINs and short PINs (PIN5, PIN6 and PIN8) (Mravec et al., 2008; Křeček et al., 2009). PIN1, 2, 3, 4, and 7 polarly localized on the PM mediating auxin directional movement from cell (layer)-to-cell (layer) under normal condition and tropic responses (Friml et al., 2003; Mravec et al.,

2008; Christie et al., 2011; Ding et al., 2011; Saki et al., 2012; Bender et al., 2013; Cazzonelli et al., 2013; Ganguly et al., 2014). PIN1 was discovered for its prominent pin-like inflorescence head, which can be mimicked by auxin transport inhibitors (Okada et al., 1991; Gälweiler et al., 1998). In the stem vasculature PIN1 was detected at the basal end of the cell and colocalized with B19 in specific PM domains mediating rootward directional auxin flow (Gälweiler et al., 1998; Blakeslee et al., 2007; Titapiwatanakun et al. 2009; Yang et al., 2013). In the L1 layer of the shoot apices, PIN1 polarity is observed dynamically rearranged during organ initiation (Reinhardt et al., 2000; Reinhardt et al., 2003; Heisler et al., 2005). *pin1* mutants exhibit fused rosette leaves, abnormal flower petals, stamens and stigma, less axillary buds, aberrant leaf lobe and misshaped caulin leaves in addition to pin-like inflorescence (Okada et al., 1991; Gälweiler et al., 1998; Reinhardt et al., 2000; Reinhardt et al., 2003). This suggests PIN1 has a unique role in shoot development, but it is not clear these abnormal phenotypes are caused by the deformation happened at the very early stage or PIN1 function is also required for the later stages. However, even the current conclusion was made based on the observation of *PIN1* expression pattern since the homozygous *pin1* mutants are infertile. *PIN1* expression was also detected in the stem and root epidermal layers, but the function remains to be determined (Blakeslee et al., 2005; Titapiwatanakun et al., 2009). PIN1 appeared also playing a role in root negative phototropic response (Zhang et al., 2014). PIN2 mediates auxin redistribution during gravitropism (Petrásek et al., 2006; Rahman et al., 2010). Mutation of *PIN3* caused alteration in phototropic bending at the hypocotyl and this suggests that other auxin transporters might be also involved (Friml et al., 2002; Christie et al., 2011; Ding et al., 2011). In the post-photomorphogenesis system, *pin3*

mutants bend at the upper hypocotyl in response to unilateral light and it indicates that other factors might regulate the initial auxin redirection during phototropism (Christie et al., 2011). Although PIN3 has been observed relocalized on the PM in the cortical cells in response to directional light, the mechanism underlies the relocalization remains to be studied. Auxin movement and related transporters in other cell layers needs to be characterized. PIN7 has been demonstrated involved in auxin redistribution at the lower part of the hypocotyl during phototropic response (Christie et al., 2011). PIN4 activity is restricted at the columella initials and columella cells and crucial for auxin reflux and to maintain the auxin gradient in the root apex (Friml et al., 2002). PIN5, PIN6 and PIN8 have been observed localized on the ER membrane regulating the subcellular auxin homeostasis and availability to nucleus signaling, which is also crucial for many aspects of plant growth and development (Mravec et al., 2009; Ding et al., 2012; Bender et al., 2013; Cazzonelli et al., 2013). PIN5 is involved in root and hypocotyl elongation process and plays essential roles in maintaining subcellular auxin homeostasis and metabolism (Mravec et al., 2009). Ectopic expression and mutation of PIN5 results in abnormal phenotypes related to auxin-dependent growth and development. PIN6 and PIN8 have also been characterized mediating many aspects of growth processes related to auxin (Ding et al., 2012; Bender et al., 2013; Cazzonelli et al., 2013). Loss-of-function and gain-of-function *pin6* mutants exhibit defects related abnormal auxin transport including nectar production, stamen/root/shoot growth, and lateral root/vascular development. PIN5, PIN6, PIN8 activities might be subjected to different regulatory mechanisms due to lacking the central long hydrophilic loop.

1.5 The Developmental roles of PIN1

Chemical inhibition of auxin transport could interfere with the normal embryo patterning (Liu et al., 1993; Friml 2003). Abolish of local auxin source interrupted the establishment of embryo axis (Robert et al., 2013). These findings suggest that localized efflux-dependent auxin gradient and auxin biosynthesis have been identified as important regulators for embryogenesis (Friml et al., 2003; Robert et al., 2013; Robert et al., 2015). From one cell stage to 16-cell stage, PIN1 signal was detected without any polarity (Friml et al., 2003). From the 32-cell stage, PIN1 starts to polarize to the basal end of the provascular cells, which correlates with the auxin gradient reverse from apical to basal. Different combinations of multiple *pin* mutants showed more severe defective phenotypes than any single mutant and this indicates that they have redundant functions (Friml et a., 2003; Robert et al., 2013). Recently, at the proembryonic stage of *wei8 tar1/2 and yuc1/4/10/11* biosynthetic mutants, *PIN1* was observed displaying apolar localization and even gradually lost signal at the later stages (Robert et al., 2013). The results suggest that localized auxin biosynthesis is required to establish PIN1 polarity and maintain *PIN1* expression during embryogenesis, but the mechanism needs further clarification (Robert et al., 2013; Robert et al., 2015). Weak signal of PIN6 has been reported in the embryo from fresh seeds, but its function in embryogenesis has not been characterized yet (Cazzonelli et al., 2013). There are also other PIN members localized on the ER-membrane. It would be interesting to uncover how the auxin in the ER participates in embryogenesis.

Auxin has been identified as a key regulator for root development. Auxin distribution displays dynamic change along with developmental events and environmental cues in the root, such as lateral root initiation, gravity stimulation, and directional light (Rahman et al., 2002; Kiss et al., 2003; Overvoorde et al., 2010; Zhang et al. 2013). Direct measurement and synthetic markers demonstrate auxin gradients and maxima existence in the root apex (Benková et al., 2003; Petersson et al., 2009). Researches from the past decades found that both localized auxin biosynthesis and PIN-mediated polarized auxin movement were crucial for root growth. All single mutation of any *PIN* gene produces almost normal root architecture, but mutations in multiple *PIN* genes could cause much more severe phenotypes in root with major alterations in auxin distribution (Blilou et al., 2005). *PIN* genes were also found expressed in distinct layers with some overlapping areas. Both imply that PIN proteins have some functional redundancy in regulating root development. In the root, PIN1 is predominantly distributed at the basal end in the vascular cells (Blilou et al., 2005). Weak signal was observed in the epidermal cells and cortex, but PIN1 function in those tissue layers remains to be characterized. In the *pin3pin4pin7* mutants, *PIN1* was detected with increased signal at the inner lateral-basal area of the endodermis and cortical cells, where *PIN3* and *PIN2* are normally expressed, respectively. Altered *PIN2* expression was also found in the *pin3pin4pin7* mutants. *PIN3, PIN4* and *PIN7* as the remaining gene in *pin* mutants, they also showed ectopic expression. The results imply that under some condition the altered expression of other *PIN* genes could rescue other *pin* mutant phenotypes. It has been reported that *PIN1* expressed in the stele and endodermal cell layer, some expression also has been detected in the cortex and epidermis at young stage

(Vieten et al. 2005). Due to overlapping functions with other PINs and *PIN1* expression in multiple layers, it still needs further elucidation how PIN1 involved in root development.

Among all the *pin* mutants, only *pin1* mutants' display pronounced defective organogenesis phenotypes including pin-shaped or occasional fasciated floral heads, fused organs, and abnormal leaf shape (Okada et al., 1991; Gälweiler et al., 1998). More evidences demonstrate that PIN1-dependent auxin canalization is a major actor for shoot organogenesis (Gälweiler et al., 1998; Heisler et al., 2005; Vieten et la., 2005; Scarpella et al., 2006; Möller and Weijers, 2009; Bender et al., 2013). In the shoot apices, *PIN1* is expressed at the L1 layer and vasculature and weak signal was detected in the L2 layer. *PIN1* expression in the L1 layer has been observed dynamically correlated with leaf and floral primordia development (Reinhardt et al., 2000; Reinhardt et al., 2003). Elevated apical-localized PIN1 signal points to the center of primordial. It suggests that PIN1 canalizes auxin into primordial meristem and depleted auxin at the peripheral zone. However the regulatory mechanism under the signal change remains to be elucidated. Later on, *PIN1* was detected basally localized at a very narrow cell file below the primordia, which was postulated as provasculature. Application of auxin transport inhibitor at the shoot apex could mimic *pin1* phenotype and exogenous auxin on the *pin1* apical part was able to induce lateral organ formation. All the data indicate that PIN1-mediated auxin flux is essential for organ position. However, whether PIN1 is required for he following organ growth has not been determined. In addition, PIN1 signal was also

detected in the hypocotyl epidermis, but the function needs further characterized (Blakeslee et al., 2005; Titapiwatanakun et al., 2009).

Two models, canalization and against the gradient, have found evidences from *PIN1* expression pattern. A canalization model, which was proposed by Sachs, mentioned that positive feedbacks between cell polarity and auxin flow leads to the vasculature differentiation and further canalize auxin away from the source (Sachs, 1991). And he also provided evidence to support this model. The studies of polar distributed membrane PIN efflux carrier proteins and computational modeling further corroborate that canalization of auxin transport holds true during many developmental processes, including vascular patterning (Scarpella et al., 2004; Sauer et al., 2006; Scanpella et al., 2006; Bennett et al., 2014; Lee et al., 2014). In against the gradient model, not only *PIN1* expression pattern is critical, but also the localized auxin biosynthesis is needed to reproduce the shoot phyllotaxis (Jönsson et al. 2006; Smith et al. 2006). It has been proposed that both models exist and this scenario nicely explains all the patterns we have observed (Bayer et al., 2009). The dynamic *PIN1* expression changes appear to play critical roles in shoot apical patterning (Gälweiler et al., 1998; Reinhardt et al., 2003; Heisler et al., 2005; Vieten et al., 2005; Möller and Weijers, 2009).

Researches about auxin biosynthesis defect mutants of *yuccas* has indicated that localized auxin biosynthesis plays an essential roles in floral patterning, vascular differentiation, normal root development, embryogenesis besides polar auxin transport (Cheng et al., 2006; Cheng et al., 2007; Robert et al., 2013; Chen et al., 2014; Zhao, 2013). Recently, a dioxygenase has been identified catalyzing auxin oxidation and

involves in reproductive development in rice (Zhao et al., 2013; Kramer et al., 2015). However, none of current models include auxin metabolism. Integration of auxin metabolism information into current models might be able to make the model more completed.

1.6 Regulation of PIN1 Activity

In *Arabidopsis*, PIN proteins as the major player for PAT have attracted considerable attention and the dynamics of canonical PIN proteins have been extensively characterized, especially PIN1. Reversible phosphorylation, cell wall, plasma membrane composition and subcellular trafficking offered flexible regulation of PIN1 polarity in response to a variety of stimulations.

1.6.1 Regulation of PIN1 activity by phosphorylation

Phosphorylation and dephosphorylation are the most common post-translational modifications of regulating protein functions or activities. Studies in the past decade have identified phosphatase and protein kinases are critical for canonical PIN proteins function and activity by regulating the central hydrophilic loop phosphorylation status (Ganguly et al., 2012). Phosphorylation and dephosphorylation interrupt PIN1 recycling between the PM and endosomes and subsequent function and activity changes. Protein phosphatase 2A (PP2A) is identified phosphatase involved in regulation of PIN dephosphorylation (Michniewicz et al., 2007). A subunit PP2AAs counteracts the activity of PINOID (PID) on phosphorylation of some serine and threonine residues in PIN central hydrophilic loop and PIN1 protein polar delivery (Michniewicz et al., 2007). Despite the broad localization, PP2AAs were observed partially colocalized with PIN1 on the PM.

Examination of *pp2aa1* double or triple mutants found that PINs polarity was changed and displayed identical manner as PIN1 polarity in PID overexpression lines (Michniewicz et al., 2007). PP2AA1 also named *ROOT CURLING IN NPA1 (RCN1)* knockout mutants showed the most pronounced defective phenotypes related to auxin transport, including root growth and root gravity response whereas *pp2aa2, pp2aa3* and *pp2aa2/pp2aa3* mutants are almost identical to wild type plants (Zhou et al., 2004; Michniewicz et al., 2007). Another two members of PP2A-C catalytic subunit, PP2A-C3 and PP2A-C4 have been reported playing essential roles in the regulation of PIN polarity and hence auxin transport and plant patterning (Ballesteros et al., 2013). Dai and his colleagues have identified another protein phosphatase 6 (PP6) whose catalytic subunits phytochrome-associated serine/threonine protein phosphatase1 (FyPP1) and FyPP3 could interact with PP2A-A in yeast and plants. Mutation of FyPP1 and FyPP3 resulted in impaired PINs polarity and disturbed auxin transport, which are similar as *pp2a-a* mutants. This led them to propose that PP2A-A and FyPP1/3 form a complex to regulate PIN1 polarity and auxin fluxes (Dai et al., 2012).

A subset of members of the AGC protein kinase family, PID, PHOT1, PHOT2, WAT1, WAT2 and D6 protein kinases (D6PKs) also have been reported functioning in auxin transport and its regulated plant development (Sakai and Haga, 2012; Santner et al., 2006; Jürgens and Geldner, 2007; Zourelidou et al., 2009). Mutational and pharmacological analysis found the PM-associated serine-threonine kinase PID, which belongs to the plant specific AGCIII family, plays a crucial role in polar auxin transport (Friml et al., 2004). The central serine residue of the conserved TPRXS(N/S) motifs

within the PIN1 hydrophilic loop have been proved as the target of PID to influence PIN1 apical-basal polarity and plant developmental processes (Huang et al., 2010). Loss of PID function abolished the auxin gradient at the shoot apical and resulted in pin-shaped inflorescence and other developmental defects, which are also shown in *pin1* mutants. Overexpression of PID could lead a basal-to-apical polarity change in PIN proteins and cause root apical meristem collapse (Friml et al., 2004; Huang et al., 2010). D6PKs are colocalized with PIN1 on the PM and studies have demonstrated that D6PKs regulate PIN1 activity through maintaining their phosphorylation status. Mutation of D6PK compromised the differential auxin transport during tropic responses and caused abnormal tropic responses (Zourelidou et al., 2009; Willige et al., 2013; Barbosa et al., 2014). Zhang et al., found modification of phosphorylation status of specific amino acid residues could change PIN1 polarity and consequent auxin fluxes (Zhang et al. 2010). Multiple kinases have been identified to regulate PIN1 activity and the associations between kinase under different developmental processes, in different tissues and environmental conditions have not been analyzed in details.

1.6.2 Regulation of PIN1 activity by subcellular trafficking

PIN proteins has been shown cycling between PM and endosomal compartments. In addition to protein kinases, there are a few other types of proteins mediate PIN cellular trafficking including ADP-ribosylation factor-guanine nucleotide exchange factor (ARF-GEF) GNOM, SORTING NEXIN1 (SNX1), vacuolar protein sorting 29 (VPS29) (Geldner et la., 2003; Jaillase et al., 2006; 2007). GNOM is a brefeldin A (BFA) sensitive protein and necessary for endosome structural integrity and function (Geldner et al.,

2003). One of weak alleles of *gnom* mutants showed pleiotropic phenotypes related to alterations of PAT. A detailed analysis found GNOM was involved in PIN1 recycling between the PM and endosomal compartment. In a BFA resistant mutational form of GNOM line PIN1 also was insensitive to BFA treatment. Recently, GNOM has been reported involved in maintaining TGN/EE function in addition to Golgi apparatus (Naramoto et al., 2014). However its substrate and action mechanism are largely unknown. An ARF GTPase activating protein (ARF-GAP) SCARFACE (SFC), which was observed mediated PIN1 trafficking in Arabidopsis, was also found played a role in AUX1 early endosome trafficking in rice (Du et al., 2011). Besides GNOM dependent auxin carrier trafficking path, a retromer protein VPS29 was illustrated essential to stabilize the retromer structure and required for proper PIN1 translocation downstream of GNOM rout during lateral root formation (Jaillase et al., 2007).

1.6.3 Regulation of PIN1 activity by cell wall and plasma membrane composition

Plant cell is enclosed by a dynamic cell wall, which provides protection and support for the cells. In addition, the cell wall structure and composition is also critical for many cellular processes. It has been observed that PIN1 polarity is highly correlated with microtubule orientation under normal and exogenous stimulations in the SAM (Heisler et al., 2010). Recently, a genetic screen for component involved in establishment and maintenance of cell polarity, a cellulose mutant *repp3* was isolated (Feraru et al., 2011). *REPP3* encodes *CESA3*, which is a catalytic subunit of cellulose synthase A. In the *cesa3* mutants, PIN protein polarity was impaired and degradation of the cell wall by pharmacological treatment mimicked the phenotypes (Feraru et al., 2011). Manditol-

induced plasmolysis and partially degradation of the cell wall also caused PIN1 protein separation from the cell wall and resulted in apolarly distribution on the PM. It suggests that connection between cell wall and PM is crucial to prevent lateral diffusion and maintain PIN1 protein polarity.

Plasma membrane consists of lipid bilayers and embedded proteins. Genetic and biochemical researches have shown that composition of lipid bilayers is playing critical roles for protein activities and functions (Tapken et al., 2015). CVP1 encodes STEROL METHYLTRANSFERASE2 (SMT2), an enzyme in the sterol biosynthetic pathway. In the *cvp1* mutants, the vascular patterning was modified and the organ expansion and elongation was abnormal (Carland et al., 2002). Studies of other *smt* mutants demonstrate that PIN protein polarity was impaired and this indicates that SMT-derived sterol implicated in maintaining PIN1 polarity and auxin transport (Carland et al., 2002; Carland et al., 2010). Very-long-chain fatty acids (VLCFAs) as a component of lipid bilayers were displayed involved in polar auxin distribution and many aspects of plant development (Roudier et al., 2010; markham et al., 2011; Yang et al., 2013). Modification the content of VLCFAs could affect the sphingolipids level. In sphingolipid deficient mutants *tsc10a-2*, ABCB19 translocation from Golgi was partially disrupted and the mutants exhibit similar phenotypes as *abcb19* (Yang et al., 2013). PIN1 and ABCB19 were observed colocalized at sterol and sphingolipids enriched micro-domains and PIN1 stability showed ABCB19-dependent manner (Titapiwatanakun *et al.*, 2009; Yang et al., 2013). However the mechanisms under the colocalization remain to be determined.

In addition to the above-mentioned factors, Auxin itself also positively regulates PIN1 gene expression (Vieten et al., 2005). Growth-created mechanical stimuli could modulate auxin distribution through impact on PIN1 function (Nakayama et al., 2012). However, the mechanisms are still not clear.

1.7 Conclusion

Auxin impacts almost every aspect of plant development. The machinery for auxin transport has been extensively characterized and a great number of elements implicated in regulation of auxin transport have been identified. There still many questions that need to be clarified. For example, recent studies have reported that function of layers covered the lateral root primordial during lateral root formation (Vilches-Barro et al., 2014). Results suggest that each layer has an active role for lateral root formation along with auxin transport and auxin signaling. Studies have observed *PIN1* is expressed in multiple domains and PIN1 plays a role in many developmental aspects. However as the consequence of severe developmental defects caused by knockout mutation, it has been difficult to conduct research using *pin1* mutants. Most of current conclusions about PIN1 function are presumably concluded based on the *PIN1* expression pattern. No reports have been published about PIN1 function in root and hypocotyl epidermis.

CHAPTER 2. DOMAIN-SPECIFIC EXPRESSION PIN1 POLAR AUXIN TRANSPORTER

2.1 Abstract

Localized directional fluxes of the plant hormone auxin play a fundamental role in embryogenesis, organogenesis and tropic growth responses. These polar streams originate in gradients generated by localized auxin biosynthesis that are strengthened, or "canalized," by a subclass of PINFORMED (PIN) auxin efflux carriers. Most prominent of these is *PIN1*. Loss of PIN1 function results in pleiotropic phenotypes including the pin-like inflorescence for which this group of genes is named. At the shoot apex, PIN1 is required for localized auxin accumulations in the L1 layer required for peripheral organ development. PIN1 also functions in root development and maintenance of the root apical meristem, although the *pin1* mutant primary roots are similar to the wild type. PIN1 also plays an important function in shoot branching, leaf venation, and leaf margin development. Additional studies indicate that auxin fluxes at different tissue-layers are playing distinct roles in plant growth and development. Results from *MERISTEM LAYER L1 (ATML1), CLAVATA3 (CLV3), FILAMENTOUS (FIL), SCARECROW (SCR), ATHB-8*, *CUP-SHAPED COTYLEDON3 (CUC3)* and *WERWOLF (WER)* promoters driven *PIN1* expression in *pin1* mutant and Col-0 indicate that PIN1 function in some domains of the SAM have dominant effects on plant growth and development.

2.2 Introduction

As the first discovered phytohormon, auxin plays instrumental roles in plant growth and development and responses to environmental cues, such as embryogenesis, organogenesis, vascular differentiation and tropic responses (Layser, 2006; Zažímalová et al., 2010). Indole-3-acetic acid (IAA) as the best characterized auxin is mainly biosynthesized at the shoot apical area and fast dividing tissues and is transported to the action sites in a polar manner by influx and efflux carriers (Ljung et al., 2005, 2013; Layser, 2006; Ikeda et al., 2009).

Research from the past decades have identified plasma membrane (PM) localized auxin transporters critical for polar auxin transport (PAT), including AUX/LAX amino acid permease with auxin uptake activities, ATP-BINDING CASSETTE superfamily B (ABCB) transporters, and PIN-FORMED proteins showing efflux activities motivated by the chemiosmotic gradient (Zažímalová et al., 2010; Grones et al., 2015). Findings in Arabidopsis indicate that ABCB1/19 are the primary auxin transporters for long-distance auxin transport from shoot apex to root (Titapiwatanakun et al. 2009; Yang and Murphy 2009; Yang et al., 2013). Some of AUX/LAX and PIN proteins asymmetrically localize in the PM in specific cell/tissue layers, which are crucial to create the differential auxin distribution (Swarup et al., 2001; Reinhardt et al., 2003; Petersson et al., 2009; Normanly, 2010; Křeček et al., 2009; Zažímalová et al., 2010; Peer et al., 2011; Adamowski and Friml, 2015). The gradients originate from localized auxin flux are playing essential roles for plant development, such as organ formation, vein differentiation and differential growth (Reinhardt et al., 2003; Tanaka et al., 2006;

Petersson et al., 2009; Pěnčík et al., 2013; Adamowski and Friml, 2015).

The major characters for localized polar auxin transport is PIN1 from the PIN protein family, which are named after the prominent pin-shaped inflorescence of *pin1* mutant (Okada et al., 1991; Gälweiler et al., 1998). Eight *PIN* genes have been isolated in Arabidopsis genome and are divided into two groups for features of the central hydrophilic loop (HL); the canonical PINs (PIN1-4, PIN7) with a long central HL and short PINs (PIN5, PIN6 and PIN8) containing a short central HL (Petrášek et al., 2009; Křeček et al., 2009; Peer et al., 2011; Adamowski and Friml, 2015). The five canonical PINs are localized on the PM with distinct and some overlapping functions. The short PINs have been characterized important for maintaining the subcellular auxin homeostasis and of which has been shown also critical for plant development (Mravec et al., 2009; Ding et al., 2012; Bender et al., 2013; Cazzonelli et al., 2013; Sawchuck et al., 2013).

PIN1 increased signal at the shoot apical meristem (SAM) has been demonstrated colocalized with auxin maxima for organ initiation (Reinhardt et al., 2003; Heisler et al., 2005). Specific expression of PIN1 in the SAM L1 layer is sufficient to recover the *pin1* mutant phyllotactic patterning defects (Kierzkowski et al., 2013). It suggests that PIN1-mediated auxin flux in the shoot apical meristem L1 layer is very important for shoot organogenesis (Reinhardt et al., 2003; Kierzkowski et al., 2013). In the root apex, PIN2-mediated shootward auxin refluxes in the epidermal and cortical cells appear to play essential roles in optimal root gravitropism (Ditengou et al., 2008; Rohaman et al., 2010; Li et al., 2015). PIN3 has been observed expressed in the hypocotyl cortical layers and

associated with hypocotyl differential growth during phototropic response (Friml et al. 2003; Ding et al. 2011). More sever phenotypes of different combinations of *pin* mutants suggest that PIN proteins have redundancies in embryogenesis, root elongation, root meristem maintenance and shoot development (Blilou et al., 2005; Benkova et al., 2003; Habets and Offringa, 2014; Adamowski and Friml, 2015). Recently, Tian and his colleagues performed a thoroughly analysis reveals that transcription factors and phytohormons such as auxin regulate aerial organ development in specific domains of the shoot apex (Tian et al., 2014). It appears that auxin fluxes in different domains have distinct functions for plant growth and development. *PIN1* expression has been observed in multiple domains and exhibits varied expression patterns along with developmental events (Reinhardt et al., 2001; Heisler et al., 2005).

We hypothesize that PIN1-mediated auxin fluxes in *MERISTEM LAYER L1 (ATML1), CLAVATA3 (CLV3), CUP-SHAPED COTYLEDON3 (CUC3)* and *FILAMENTOUS FLOWER (FIL)* domains would affect shoot development and tropic responses, in *Homeobox gene 8 (ATHB-8)* domain would complement *pin1* altered vein patterning and in *SCARECROW/SCR* and *WEREWOLF (WER)* domains would impact root growth and tropic responses. In an effort to decipher PIN1 function in different domains, we determined to control *PIN1-GFP* expression by domain-specific promoters (Table1). Our imaging results found that domain-specific promoters are not as specific as they have been reported. *proATML1, proCLV3* and *proCUC3* expression in *pin1* mutants could not fully complement *pin1* phenotypes. *proSCR::PIN1-GFP* expression in Col-0 could cause aberrant phenotypes, but the reason is not clear.

2.3 Results

2.3.1 *proATML1::PIN1-GFP* expression pattern and complemented phenotypes

Studies of PIN1 expression in shoot apex suggested that PIN1 in the L1 layer played an instructive role in leaf and floral precordium development and phyllotactic patterning (Reinhardt et al., 2001; 2003). We hypothesize that *PIN1* expression in the L1 layer would be able to complement *pin1* shoot developmental defects. In the aim to decipher PIN1 function in SAM L1 layer, we determined to express PIN1 driven by *MERISTEM LAYER L1 (ATML1),* which specifically marks the SAM L1 layer and some expression at the primary root and lateral root has been reported (Sessions et a., 1999).

We selected the transgenic lines showing SAM L1 specific expression pattern (Figure 2.2) and the T-DNA insertion in *PIN1* gene. The *PIN1* expression largely recapitulated the publish data during the seedling stage. In *proATML1::PIN1-GFP* line, the signal was observed in SAM L1 layer including the hypocotyl and cotyledon epidermal cells and showed some expression at the primary root vascular (Figure 2.2A, B, C) . Earlier studies have shown that PIN1-mediated auxin flux in the shoot apical tissue is critical for plant organogenesis (Reinhardt et al., 2005), especially the high level of auxin plays a key role for primordia specification and organ growth (Reinhardt et al., 2003). We further identified the *proATML1::PIN1-GFP* lines with *pin1* homozygous background. In the confirmed plants, predominant phenotypes previously observed in *pin1* mutants were observed, including solely pin-formed inflorescences, an occurrence of a fasciated inflorescence, and partially curled or twisted rosette and cauline leaves

(figure 2.2) (Gälweiler, 1998; Vernoux et al., 2000). However, ectopic *PIN1* expression in the SAM L1 does not create any defects in Col-0 plants (Figure 2.2).

Our work found that *proATML1::PIN1-GFP* expression did not complement the *pin1* phenotypes as previously published (Kierzkowski et al., 2013). It appears that we used a 3.0kb *ATML1* promoter region instead of 3.9kb region, which was used by Kierzkowski. We also noticed that Kierzkowski has been monitoring *PIN1* expression at every stage to make sure *PIN1* expressed in the L1 layer and only those transgenic plants containing *PIN1* expression in L1 layer at all the stages could be complemented (Kierzkowski et al., 2013). We only checked the PIN1 signal at the young stages. We still need to confirm *PIN1* expression in other stages.

2.3.2 *proCLV3::PIN1-GFP* expression domains and recovered pin1 phenotypes

PIN1 is strongly expressed in the tunica and loss-of-PIN1 function caused a needle-like inflorescence and reduced auxin transport in the inflorescence (Okada et al., 1991; Vernox et al., 2010). We hypothesize that *PIN1* expression in the tunica might be able to complement *pin1* inflorescence defects, or at least partially complement the defects. In order to test this hypothesis, we determined to use *CLAVATA3 (CLV3)* to drive *PIN1* expression in *pin1*. *CLV3* has been detected highly expressed in central stem cell region in the SAM L1 layer with lesser degree in sub-epidermal L2 layer (Fletcher et al., 1999; Brand et al., 2002). The expression could expand to the rib meristem at later stages (Yadav st al., 2014).

However, in the transgenic line that *proCLV3::PIN1-GFP* was expressed in *pin1* homozygous plants, the plants produced needle-like inflorescences just as *pin1* (Figure 2.3A, B). Similar abnormal rosette leaves and cauline leaves were seen in the transgenic plants. Additionally, previously observed flower defects were observed, with all flowers present being deficient in number of petals and lacking stamens (Okada et al., 1991). A stray flower on a secondary inflorescence has been occurred in the *proCLV3*::PIN1-GFP line once and produced less than twenty seeds. 6-week old wild type plants with *proCLV3::PIN1-GFP* transgene grew totally normal (Figure 2.3C).

The work here presented that *proCLV3::PIN1-GFP* was not able to complement the *pin1* inflorescence defects. Earlier studies have performed genome analysis and shown that the CZ contains less auxin and GA-responsive genes (Yadav et al., 2014). These may explain why the *proCLV3::PIN1-GFP* could not complement *pin1* defects (Figure 2.3B). The auxin transport in the transgenic line has not been analyzed.

2.3.3 *proCUC3::PIN1-GFP* expression pattern and complemented pin1 phenotypes

Without PIN1 activity, *Arabidopsis* forms less lateral organs and results in needle-like inflorescence (Okada et al., 1991). PIN1-mediated auxin maxima have been shown critical for leaf positioning (Reinhardt et al., 2003). Auxin efflux and influx-mediated auxin minimum is required for axillary meristem initiation (Wang et al., 2014a; Wang et al., 2014b). As the primary mediator of auxin polar transport in the SAM, we hypothesize that specifically expression of PIN1 in the boundary region might be able to make *pin1* mutants produce normal lateral organs, such as leaves and flowers. The NAC domain proteins CUC1, CUC2, and CUC3 has been found enriched in the boundary

zone of the shoot apex, however only *CUP-SHAPED COTYLEDON3 (CUC3)* plays essential roles in axillary meristem initiation and development (Vroemen et al., 2003; Hibara et al., 2006; Yang et al., 2016). In the aim to decipher PIN1 function in the boundary zone, we determined to use *CUC3* to drive PIN1-GFP expression in *pin1* mutants.

We identified two transgenic lines carrying T-DNA insertion in the *PIN1* gene for further analysis. Both wild type and *pin1* homozygous plants with *proCUC3::PIN1-GFP* expression have been imaged (Figure 2.4A, B). One line with *pin1* homozygous background has pin-like inflorescence and less secondary inflorescence (Figure 2.4C). Although the other line also has pin-like inflorescences, the plant developed more secondary inflorescences (Figure 2.4D). The PIN1-GFP overexpression in the boundary region does not interfere the col-0 growth (Figure2.4E).

It appears that both lines still developed pin-like inflorescence head. This suggests that *CUC3::PIN1-GFP* expression *pin1* could not rescue the floral defects. However, the increased secondary inflorescence in one transgenic line indicates that *CUC3::PIN1-GFP* expression might be able to complement the lateral organ initiation other than the flower initiation and development. It still needs more plants to confirm the results.

2.3.4 *proSCR::PIN1-GFP* ectopic expression pattern and resulted pin-like inflorescence in Col-0

Aside from the critical roles of PIN1-mediated auxin flux in the shoot organogenesis, Auxin stream in the endodermis has been shown related to the hypocotyl phototropic responses and lateral root formation (Ding et al., 2011; Vilches-Barro et al., 2014). To

further decipher PIN1 function in the endodermal layers, we determined to express *PIN1-GFP* under control *SCARECROW (SCR)* promoter, which is an endodermis specific gene, and strong expression in the shoot apex (Di Larurenzio et al., 1996; Malamy et al., 1997). We expect that PIN1-GFP expression driven by *proSCR* might be able to complement *pin1* shoot developmental defects, since *SCR* strongly expressed in the shoot apex.

In the *proSCR::PIN1-GFP* transgenic lines, *PIN1-GFP* expression largely recapitulated the publish data, however weak signal was also showed in the neighboring cortical cells (Figure 2.5A, B). Unfortunately, we have not identified the line with *pin1* homozygous background yet. However the wild type plants with *proSCR::PIN1-GFP* ectopic expression displayed varied phenotypes from normal to *pin1* mutant like phenotypes (Figure 2.5D, E, F). While rosette leaf defects in some plants are immediately evident and then followed by pin-formed inflorescences, in other plants rosettes are similar to Col-0 and inflorescence growth defects are minimal or non-existent. Among the plants that show a severe phenotype there is also immense variation. While some generate only pin-formed inflorescences and no siliques or seeds, others produce initial pin-formed inflorescences, but secondary inflorescence growth will dominate and these will produce normal flowers and abundant siliques and seeds.

Although, we have not been able to identify the transgenic line with *pin1* background yet, the aberrant phenotypes caused by PIN1 ectopic expression suggests that the SCR driven PIN1 expression could interrupt directional auxin movement in the shoot apex. Further analysis is needed to characterize the impacts of *proSCR::PIN1-GFP* expression on the *pin1* development.

2.3.5 *PIN1-GFP* expression driven by proATHB-8

Polar flow of auxin is very important for plant vascular formation and patterning (Sachs, 1991). PIN1 and its related family members appear to be essential for auxin polar transport, especially PIN1 (Adamowski and Friml, 2105). In *pin1* mutants, enhanced vascular tissue differentiation right below the young leaves has been observed and this suggested PIN1-mediated auxin transport out of the source is necessary for vascular tissue development (Okada et al., 1991). We hypothesize that PIN1 specific expression in the vascular tissue might be able to complement *pin1* vascular alterations. In the aim to test this hypothesis, we determined to use *ATHB-8* promoter, which is a procambium marker with discontinuous expression at the shoot apical stem region, to drive *PIN1* expression in *pin1* (Kang et al., 2003; Yadav et al., 2014).

In the young seedlings of *pATHB-8::PIN1-GFP,* signal showed in the cotyledon epidermis and leaf vein in the shoot and strong florescence signal was also detected in the root procambial cells and quiescent center (Figure 2.6A, B, C) (Kang et al., 2003). So far, we are still working to isolate the plants with *PIN1* knockout background. The Col-0 plants with *PIN1* ectopic expression in those domains did not display any abnormal phenotypes (Figure 2.6D).

2.3.6 *PIN1-GFP* expression driven by proFIL

The *pin1* mutants barely produce any functional flowers and this indicated that PIN1 has an essential role for flower development (Okada et al., 1991). To further test PIN1 function for inflorescence development, we determined to express *PIN1* controlled by *FILAMENTOUS (FIL) FLOWER* promoter, which is a transcription factor required for inflorescence and floral development. Strong expression has been detected at the abaxial side in the leaf and floral primordial (Sawa et al., 1999).

In the seedling stage, PIN1-GFP was observed in the cotyledon petioles and a narrow central region in the primary root in the five-day old seedlings (Figure 2.6E, F). The ectopic expression of *proPIN1::PIN1-GFP* does not cause any abnormal phenotypes in Col-0 (Figure 2.6G). However, more work needs to be done to isolate the *pin1* mutants with *proPIN1::PIN1-GFP* expression.

2.3.7 *PIN1-GFP* expression driven by proWER

In the young stage, *PIN1* expression has been detected in the root epidermal cells (Vieten et al., 2005). However, *pin1* mutants have largely normal root system. No one has ever reported PIN1 function in the root epidermis. In order to decipher PIN1 function in the root epidermis, we determined to use *WERWOLF (WER)* promoter, a non-root hair cell-type marker to control PIN1 expression in *pin1* mutants. *WER* was found expressed in shoot apex, leaves, stems, floral buds and mature flowers (Ryu et al., 2005; Seo et al., 2011).

In the *proWER::PIN1-GFP* transgenic lines, *PIN1-GFP* was expressed in shoot apex and root apex and very weak signal was detected in the vasculature (Figure 2.6H, I) (Ryu et al., 2005; Seo et al., 2011). So far we only identified lines with Col-0 background, and *proWER::PIN1-GFP* ectopic expression does not pose any visible impact on the plant normal growth (Figure 2.6J).

2.4 Discussion

The shoot apicex has been divided into a central zone (CZ), located at the summit of the SAM, a peripheral zone (PZ), to initiate the lateral organs and maintain their growth and polarity (Vernoux et al. 2010). Studies of Reinhardt and his colleagues suggested that PIN1-mediated auxin fluxes are essential for organogenesis (Reinhardt et al., 2000; Reinhardt et al., 2003; Reinhardt et al., 2005). In the root, PIN1 has some roles in root development overlapped with other auxin transporters. Here we introduced *PIN1-GFP* into *pin1* mutants driven by domain-specific promoters in order to assess the impacts *PIN1* expression in specific tissue layers. However our visualization of *PIN1-GFP* revealed that the cell type-specific promoters are not as tight as they have been reported (Sessions et a., 1999; Fletcher et al., 1999; Brand et al., 2002; Sawa et al., 1999; Di Larurenzio et al., 1996; Malamy et al., 1997; Kang et al., 2003; Yadav et al., 2014; Vroemen et al., 2003; Hibara et al., 2006; Ryu et al., 2005; Seo et al., 2011). In the *proATML1::PIN1-GFP* lines, signal was also detected in the root vasculature in addition to the SAM L1 layer (Figure 2.2C). CUC3 is often used as a marker for the boundary region, *proCUC3::PIN1-GFP* lines also showed signal in the vascular of RAM (Figure 2.4B). Given the reported tissue layers, the cotyledon epidermis contained GFP signal in

the *proATHB-8::PIN1-GFP* transgenic plants (Figure 2.6A). Stronger signal was detected in the vascular of the *proFIL::PIN1-GFP* lines (Figure 2.6F). Weak signal was shown in the neighboring cortical cells when endodermis-specific promoter SCR drove PIN1-GFP (Figure 2.5B). As for *proWER::PIN1-GFP* lines, very weak signal also could be detected in the vasculature in the root apex (Figure 2.6I). The unexpected expression domains in those domain-specific promoters suggest that when using those promoters to study gene function in specific cell-types, it must be careful to interpret the results.

Our work found that with *proATML1::PIN1-GFP* expression, *pin1* mutant was still grown like *pin1*, instead of being complemented (Kierzkowski et al., 2013). We found that we used shorter ATML1 promoter and we also did not monitor *PIN1* expression in different stages to make sure its expression in the L1 layer. Genome analysis has shown that the CZ contains less auxin and GA-responsive genes (Yadav et al., 2014). These may suggest why the *proCLV3::PIN1-GFP* does not complement *pin1* defects (Figure 2.3B). It has been reported that the boundary zone cells (the region between central and peripheral zones) could differentiate into both types of cells (Gordon et al., 2009). We observed more secondary inflorescences in one of the *proCUC3::PIN1-GFP* lines (Figure 2.4D). However, it is difficult to determine the severity of phenotype, as there is significant phenotypic plasticity in the *pin1-7* line and the sample sizes were small due to the fact that all lines are maintained as heterozygous. Additionally, the phenotype may vary by light treatment.

Among all the domain-specific promoter lines, *proSCR::PIN1-GFP* expression in Col-0 particularly resulted in dramatic phenotypes (Figure 2.5D, E, F). The severity of

phenotype varied among the individuals within the *proSCR::PIN1-GFP* lines. Previously reported PIN1 mutant phenotypes were observed, such as multiple silique growth from one position, multiple secondary inflorescence growth from a single position, asymmetric rosette leaves, and phyllotaxis defects (Huang et al., 2010; Lampugnani et al., 2013; Zhang et al., 2009). This might cause by auxin accumulation in specific regions, but it still needs further characterization.

2.5 Material and Methods

Plant Material

Mutants and wild type are all in the standard Columbia (col-0) background. *pin1* mutant used in this study is SALK_047613 and maintained in heterozygous condition since homozygotes do not make any viable seeds. For mutants, homozygous plants could be isolated through phenotypes; heterozygous plants were identified by genotyping. primers used for genotyping are listed in table 2. For heterozygous plants, PCR products contained a shorter fragment (approximately 800 base pairs [bp]) and a longer fragment (approximately 1200 bp), for homozygous plants, the PCR products only had the shorter fragment and for wild type plants, the PCR product only had the longer fragment.

T0 transgenic seeds were sowed in the soil and five days after germination, 0.1% basta was sprayed every day until only the positive transformants were survived. T2 seeds were used to perform the analysis. Transgenic plants were genotyped to confirm *pin1* homozygous background and the transgene of PIN1-GFP. For the wild type, the PCR product is 2100 bp. For the plants with PIN1-GFP transgene, the PCR product is 2800 bp. The following primer pairs were used: LBb1.3 5’ TTTGCCGATTTCGGAAC-

3’ and PIN1R 5’-CTTGGGTTTAACGCCATGAACA-3’to verify T-DNA insertion. To verify for PIN1-GFP primers flanking the GFP insertion site were chosen: PIN1F 5’-TTTGATCTCCGAGCAGTTTCCA-3’and 5’- CTTGGGTTTAACGCCATGAACA-3’.

Plant Growth Conditions

For seedlings used for confocal imaging, seeds were surface-sterilized with 10% bleach for 10 minutes and rinsed with autoclaved water 5 times. Then seeds were soaked in water and put into fridge (4°C) overnight for stratification. Seedlings were grown on 0.8% phytoblend plates, containing 1/4 MS basal salts, 0.5% sucrose, pH 5.5, at 21-23°C, with 16 h of daylight at 100 μmol m^{-2} s^{-1}. For the adult plants used for phenotyping, seeds were sterilized in 70% ethanol with agitation 3 minutes, rinsed with 95% ethanol, and dried on filter paper. Sterile seeds were plates on ¼ Murashige & Skoog medium, 0.5% sucrose, 0.8% agar, 10mg/L Basta plates, cold treated at 4˚C 24 hours, then grown under continuous 80 μM $m^{-2}s^{-1}$. 5d seedlings were transferred to soil and grown at 22˚C under 80-100μM $m^{-2}s^{-1}$ light for 16 hour days at 40% humidity.

Construction of transgenes and Plant Transformation

The single-site Gateway®-compatible pEarleyGate 100 was used as a backbone and the 35s promoter before the gateway cloning cassette in pEarleyGate 100 was removed by PCR to generate pEarleyGate 100Δ (Earley et al., 2006). The domain-specific promoters were selected to regulate the PIN1 expression (Table1). The PIN1 gene with the three-prime untranslated region (3'-UTR) was amplified from the wild type (col-0) genomic DNA and then sub-cloned into pEntr-D-topo vector (Heisler et al., 2005). The GFP tag was integrated into *PIN1* through Infusion-PCR and could facilitate

the visualization of where *PIN1* expression. Finally, PIN1-mGFP was assembled right after the domain-specific promoters through LR reaction (Fig.2.1) (Spiliotis, 2012). All the primers used were listed in table 2. Constructs were verified through sequencing. Primers for sequencing are listed in the supplemental table3. Destination vectors were transformed into the Agrobacterium strain Gv3101. Heterozygous *pin1*$^{-/+}$ plants were used for transformation through floral dipping transformation (Clough et al., 1998).

Microscopy

Seedlings were observed under the Zeiss LSM710 confocal microscope to confirm the PIN1 expression. Primary roots and shoot apex were imaged. The florescence signal was collected with separate channels under the same track setting. For chlorophyll, 594 nm (0.2%) excitation and 647–721 nm emission. For GFP: 488 nm (20%) excitation and 493–571 nm emission. Images were using Zen 2009 software and Photoshop CC software (Adobe Systems).

Table 1. Domain-specific promoters for controlling *PIN1* expression
Promoters were selected from an examination of the literature. CZ, central zone.

Tissue	Cell type	Gene	Locus
SAM	L1	*MERISTEM LAYER L1 (ATML1)*	At4g21750
	L1-L3 CZ	*CLAVATA3 (CLV3)*	At2g27250
	SAM/LP border	*CUP-SHAPED COTYLEDON3 (CUC3)*	At5g53950
Leaf primordia	P0, abaxial P1/2	*FILAMENTOUS FLOWER (FIL)*	At2g45190
Provasculature	Protophloem, abaxial, P1/2	*HOMEOBOX PROTEIN25 (ATHB25)*	At5g65410
	Procambium/(xylem)	*Homeobox gene 8 (ATHB-8)*	At4g32880
Bundle sheath	Endodermis	*SCARECROW/SCR*	At3g54220
Epidermis	None root hair forming	*WEREWOLF (WER)*	At5g14750

Table 2. Primers used to genotype *pin1-7,* amplify domain-specific promoters and PIN1-GFP constructs.

Primer	Sequence
LB.b 1.3	ATTTTGCCGATTTCGGAAC
PIN1R	CTTGGGTTTAACGCCATGAACA
PIN1F	TTTGATCTCCGAGCAGTTTCCA
GFP-F	ATGGTGAGCAAGGGCGAGGA
GFP-R	TTCTGCTGGTAGTGGTCGGCGA
PIN1-LP	CAAAAACACCCCCAAAATTTC
PIN1-RP	AATCATCACAGCCACTGATCC
proSCR-F	CTCCCTCGAGTCGAGATCACAAGTTTGTACAAAAAAGCT
proSCR-R	GTGATCTCGACTCGAGGGAGATTGAAGGGTTG
proATHB8-F	AAAGCTCGAGTCGAGATCACAAGTTTGTACAAAAAAGCT
proATHB8-R	GTGATCTCGACTCGAGCTTTGATCCTCTCCGATCT
proATML1-F	TCCACTCGAGTCGAGATCACAAGTTTGTACAAAAAAGCT
proATML1-R	GTGATCTCGACTCGAGTGGATTCAGGGAGTTTCTTT
proCUC3-F	AAAGCTCGAGTCGAGATCACAAGTTTGTACAAAAAAGCT
proCUC3-R	GTGATCTCGACTCGAGCTTTTACTTAATATAACTGAAAAAG AGACT
proWER-F	AGACCTCGAGTCGAGATCACAAGTTTGTACAAAAAAGCT
proWER-R	GTGATCTCGACTCGAGTCTTTTTGTTTCTTTGAATGATAGAC G
proCLV3-F	CTCTCTCGAGTCGAGATCACAAGTTTGTACAAAAAAGCT
proCLV3-R	GTGATCTCGACTCGAGAGAGAGAAAGTGACTGAGTG
proFIL-F	AAAGCTCGAGTCGAGATCACAAGTTTGTACAAAAAAGCT
proFIL-R	GTGATCTCGACTCGAGCTTTTTTGTAAGAAGGGGAAA
mGFPF(PIN1Fusion)	CTGATATTTACTCGAGAATGAGTAAAGGAGAAGAACT
mGFPR(PIN1Fusion)	CTTGAGACCTCTCGAGTTTGTATAGTTCATCCATGCC
pEntr-PIN1LF	AAAGATGATTACGGCGGCGGACTTCT
pEntr-PIN1LR	GGCGCGCCGGTGAAGGGGGCGGCCGC
pEntr-PIN1-F	AAAGATGATTACGGCGGCGGACTTCT
pEntr-PIN1-R	GGCGCGCCGGTGAAGGGGGCGGCCGC

Table 3. Primers used to sequence domain-specific promoters and PIN1-GFP constructs.

Primer	Sequence
CUC3-25	CTTACCTTTGCAAGAATTCT
CUC3-166	GCAGTTTTTACTCAGCTTTA
CUC3-726	AAGTAGTAATTTAAAAGAAGAAAA
CUC3-866	TTACATTCCTTTCTTTGTTAC
CUC3-1427	GACATGAATATGCATAGTCG
CUC3-1566	CGAAGCCTTCATAAATATGG
CUC3-2128	GTTTCTCCACCTTCTCCTTC
CUC3-2267	TTAAAAAATTTCAGAACACA
CUC3-2829	CGTACGAGTACAAAAGATTTCCAAA
CUC3-2968	TGCCCAAGTGATACCATCGG
CUC3-3530	AAACATTTAATATCGACAATAT
CUC3-3688	GTTTCGGTTTTGACACATAT
CUC3-4231	AGTTCAACTAGTCTGGATCA
CUC3-4394	GGATACTGTTTTGGGTGTATATTAG
CUC3-4932	CACTCCATTTTCTCTCTCTAT
CUC3-5077	TCTTTTACTTAATATAACTGAAAA
proATHB-8-600F	GAATTCAATACTTGTTTTGTT
proATHB-8-1200R	CTGGTCTCTGGAAGCAAAGG
proATHB-8-1200F	CCTTTGCTTCCAGAGACCAG
proATML-750F	TTGATCCATAATTCTTTTCG
proATML-850R	GATTAGACAGTAGTACCCCA
proATML-1450F	CGTATATGATGTAGGTGATGTA
proATML-2150F	TTTTGAGATTAAATTGTTTAGT
proATML-2500F	AATCTAAAATGAACTATAACCA
CLV3-925F	CGTATACATACTGTTGTATTA
FIL-610F	GCCTCAATCTTCCCTTGTTCATAC
FIL-1202F	AACTAATGTATGTTTATTTTATCGAT
WER-740F	ATTGCCTCAACTTCCAGC
WER-1360F	CCGTGAAGAATCCTAATCCC
WER-1820F	CAAGAACCTTCACAACCTCC
PIN12505F	TAAGTTCCAAGATGAGCG
PIN13845F	CAACGGTTCTTTAGATTGG
PIN14373F	CTCTAACTTTGGTCCTGG
PIN14998F	GTAACAAAGACGATGATAGC
PIN15456F	CTATGTTCAGTCTTGGTCAGTT
PIN16265F	TTGCCATTATCCAGGTAG
PIN16816F	GAAGAGAAGAAGAAGGAGAC
PIN17444F	ACATACCACTTAGATGCTG
PIN17997F	GAGAGAAGAAGAAGACGC
PIN18426F	GATGTTAGTGTCTCTGCC

Continuation of Table 3.

PIN18831F	CATTACACTGCTCCATCG
PIN13591R	AACCATAGCCGTCATAAC
PIN14179R	AGCATTAGAACGACGAAC
PIN14791R	GTTACTATTCCCCTGAGG
PIN15460R	GAACTGACCAAGACTGAAC
PIN16153R	TCTTCTGTTTCCACAAGC
PIN16846R	AGGTAAAACTTGCTGAGC
M13 reverse	AGCGGATAACAATTTCACACAGG
M13 Forward	CCCAGTCACGACGTTGTAAAACG

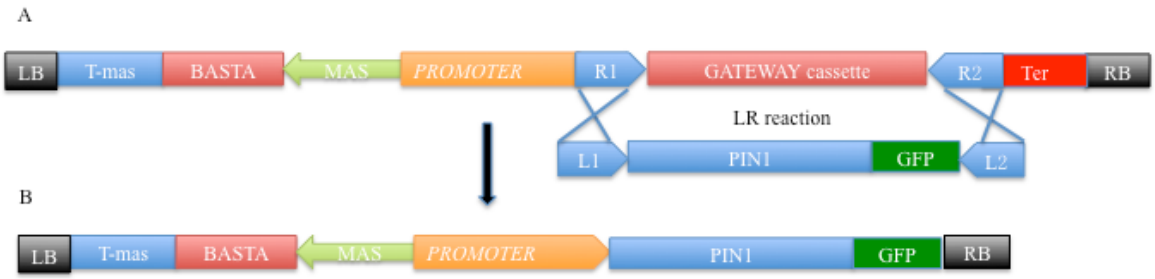


Figure 2.1. **Schematic diagram of single site Gateway® cloning destination vectors with tissue-specific promoters for PIN1 stable expression with Basta resistance selectable marker.**

(A) The domain-specific promoters were cloned into the gateway binary vectors right in front of the gateway cassette by In-Fusion cloning PCR to create a domain-specific destination vector (Spiliotis, 2012). (B) PIN1-GFP (gene of interest) is recombined with the destination vector through LR reaction.

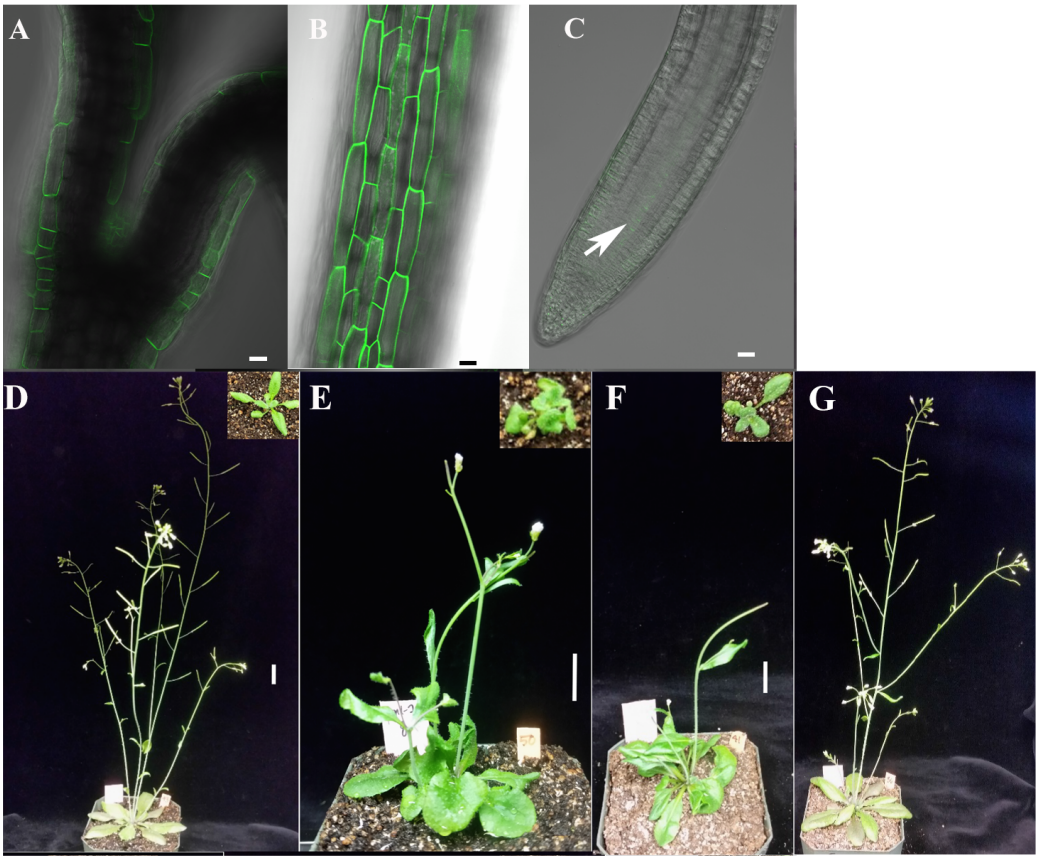


Figure 2.2. *proATML1::PIN1-GFP* expression pattern and resulted phenotypes.

(A) *proATML1::PIN1-GFP* in the shoot, (B) in the hypocotyl and (C) weak signal could be observed root. (D) Mature plants col-0, (E) *pin1* homozygote, (F) *pin1* with *PIN1-GFP* expression driven by ATML1 promoter. (G) Col-0 with *proATML1::PIN1-GFP* ectopic expression has normal inflorescence. Arrowheads point to the weak signal area. Bars equal to 20µm. Violet color shows chlorophyll autofluoresence in all the images. Seedlings from T2 seeds were visualized with zeiss LSM710 confocal microscope. 6-week old plants were photographed.

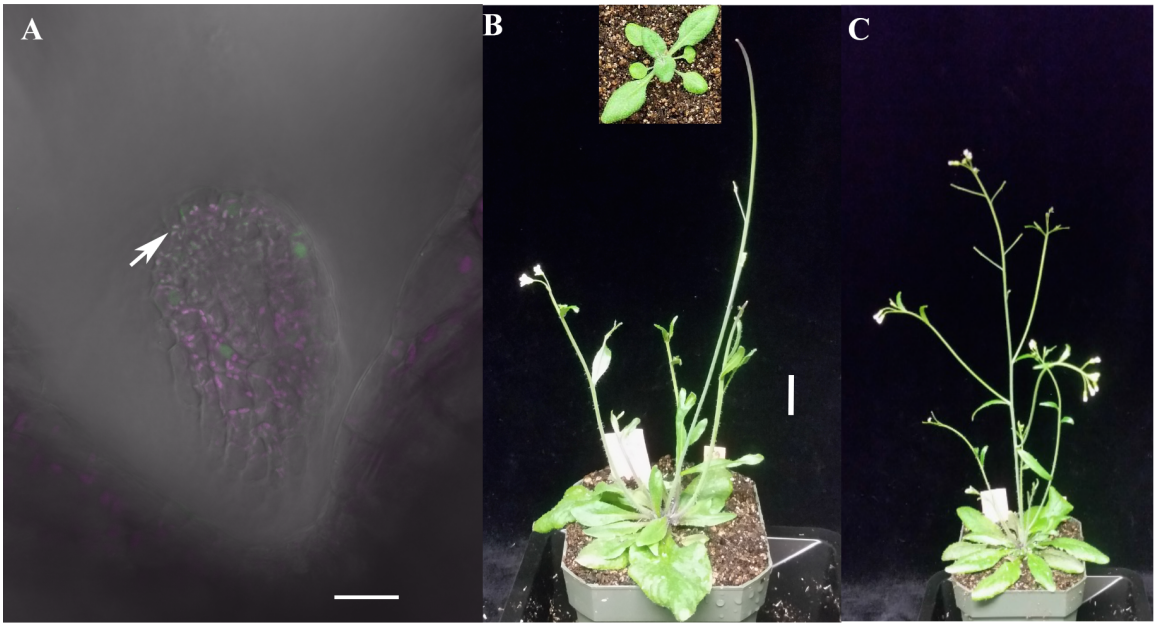


Figure 2.3. *proCLV3::PIN1-GFP* expression pattern and plants phenotypes.

(A) *proCLV3::PIN1-GFP* in the SAM. (B) 6-week old *pin1*plants with *PIN1-GFP* expressed driven by CLV3 promoter. (C) Col-0 with *proCLV3::PIN1-GFP* ectopic expression grows normal. Arrowheads point to the signal region. Bars equal to 20µm. Violet color shows chlorophyll autofluoresence in all the images. T2 seeds were germinated on plates and five-day after germination seedlings were visualized with zeiss LSM710 confocal microscope.

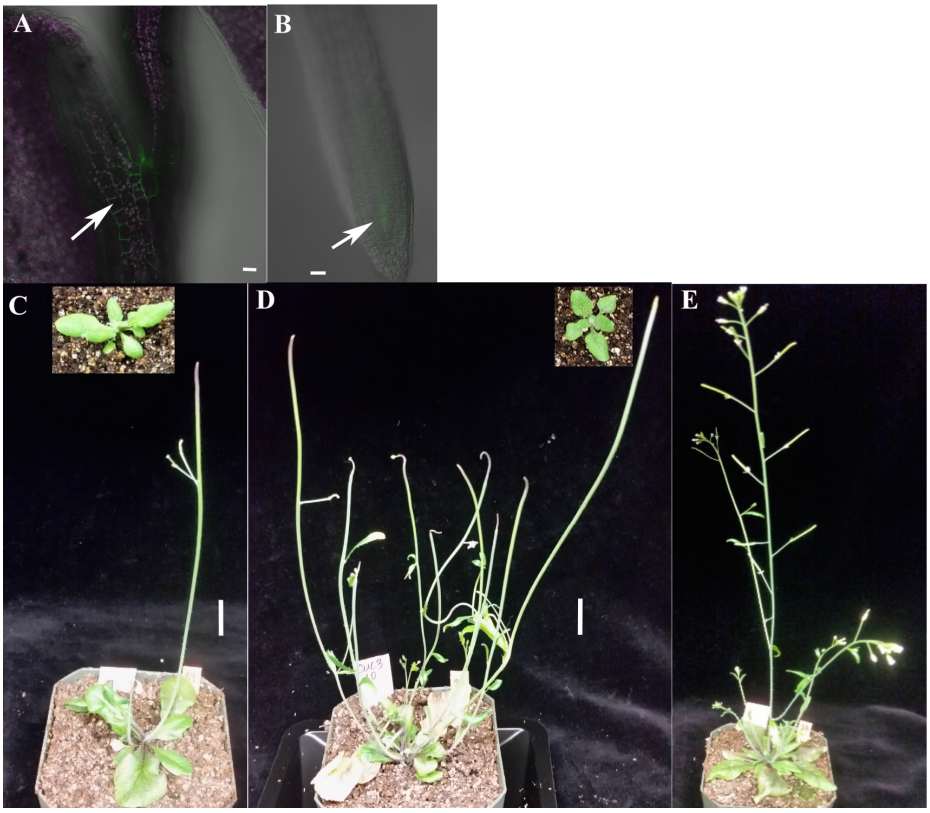


Figure 2.4. *proCUC3::PIN1-GFP* expression pattern and plants phenotypes.

(A) *proCUC3::PIN1-GFP* in the shoot, (B) in the root. 6-week old *pin1*plants with *PIN1-GFP* expressed driven by CUC3 promoter have needle-like inflorescence (C) line 1 and (D) line10. (E) Col-0 with *proCUC3::PIN1-GFP* ectopic expression grows normal. Arrowheads point to the signal region. Bars equal to 20µm. Violet color shows chlorophyll autofluoresence in all the images. T2 seeds were plated and five-day after germination seedlings were visualized with zeiss LSM710 confocal microscope.

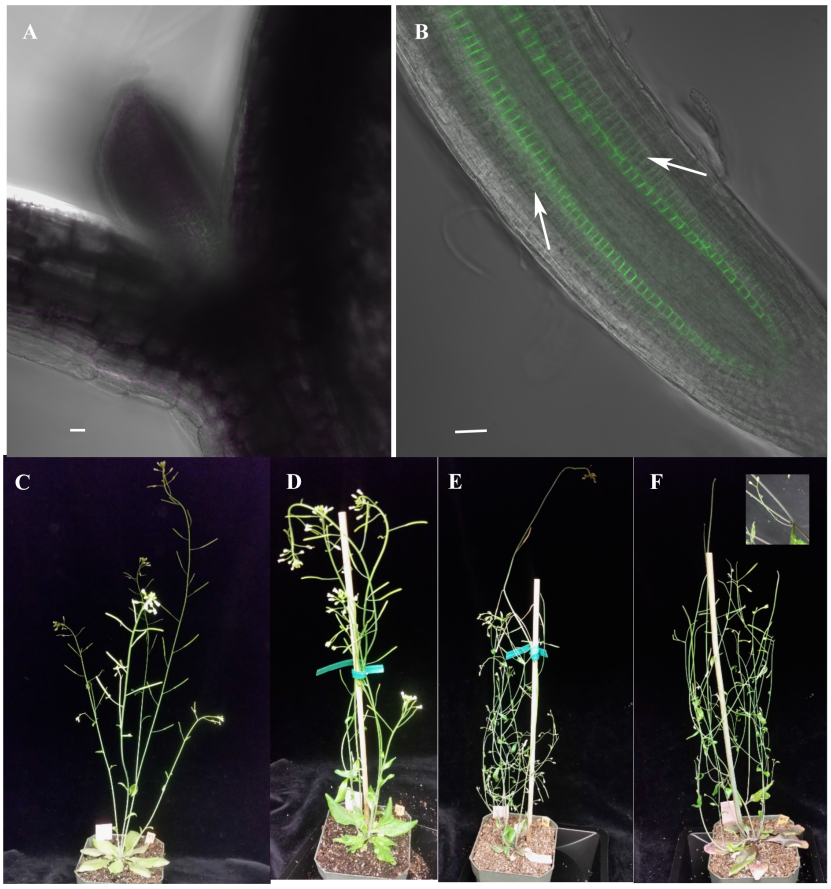


Figure 2.5. *proSCR::PIN1-GFP* ectopic expression in col-0 and plants phenotypes.

(A) signal of *proSCR::PIN1-GFP* in the shoot and (B) in the root endodermis and weak signal was observed at the adjacent contex cells. 6-week old col-0(C), col-0 with *proSCR::PIN1-GFP* ectopic expression, plants have varied phenotypes ranging from wild type to pin-like inflorescences and abnormal rosette leaves (D), (E), (F). Arrowheads point to the signal region. Bars equal to 20µm. Violet color shows chlorophyll autofluoresence in all the images. T2 seeds were grown and five-day old seedlings were visualized with zeiss LSM710 confocal microscope.

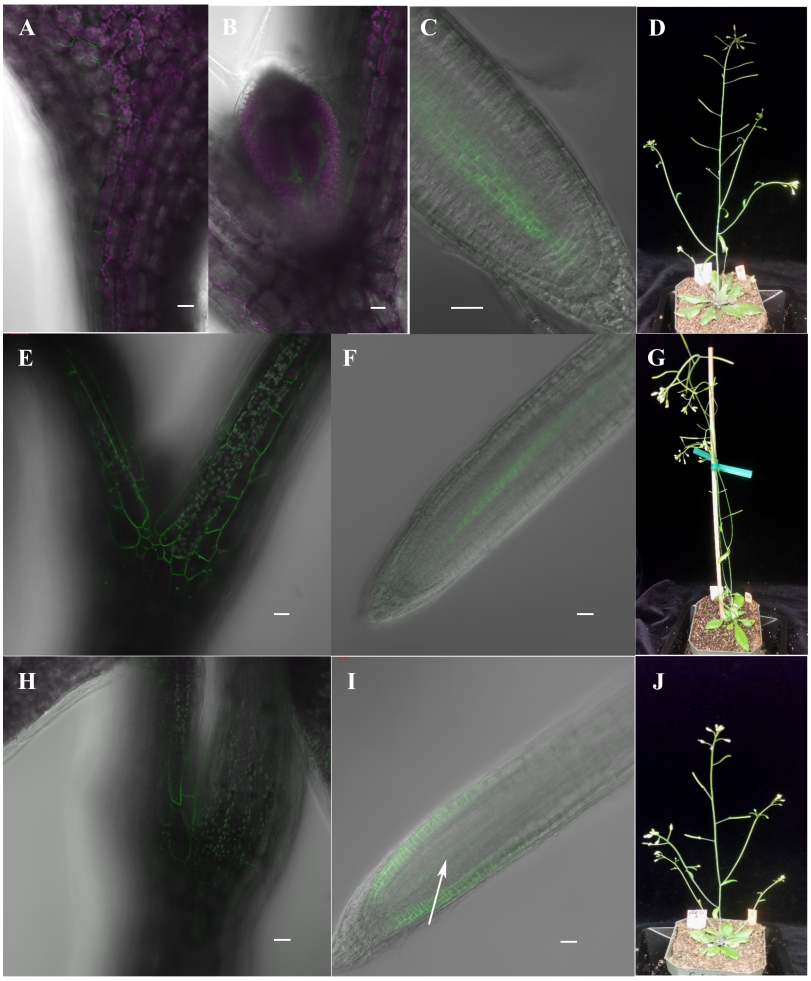


Figure 2.6. *PIN1-GFP* expression driven by *ATHB-8, FIL* and *WER* promoters and plants phenotypes.

proATHB-8::PIN1-GFP in cotyledon epidermis (A), in the leaf vein (B) and in the root procambium cells (C). Mature col-0 grows normal with *proATHB-8::PIN1-GFP* expression (D). *proFIL::PIN1-GFP* in cotyledon petiole (E), weak signal in the root vasculature (F). Mature col-0 with *proFIL::PIN1-GFP* expression has less secondary inflorescences (G). *proWER::PIN1-GFP* in the shoot epidermis (G), in the root epidermal cells (H), weak signal in the root vasculature (I). Mature col-0 with *proWER::PIN1-GFP* expression is normal (J). Arrowheads point to the signal region. Bars equal to 20µm. Violet color shows chlorophyll autofluoresence in all the images. Five-day after germination seedlings were visualized with zeiss LSM710 confocal microscope.

CHAPTER 3. EXPLORING PIN1 FUNCTION BY INDUCIBLE DOMAIN-SPECIFIC EXPRESSION SYSTEM

3.1 Introduction

Localized auxin movement has been found important for phototropic responses, lateral root formation and shoot organogenesis. *PIN1* as the primary polar auxin movement mediator has been observed expressed in multiple domains in *Arabidopsis* (Benková et al., 2003). However it has been difficult to study PIN1 function in its mutant background since the homozygous *pin1* plants are infertile. As most of the conclusions about PIN1 function are postulated based on its expression pattern. The *pin1* mutants are sterile and exhibit defectives at different developmental stages. In order to separate the abnormal phenotypes of one stage from the other stages, we considered to use an domain-specific inducible system to spatially and temporally control *PIN1* expression. Before you convert to PDF, carefully review our Sample Thesis Pages and our Formatting Checklist, and then double check the formatting of your entire document, page by page.

The inducible systems such as ethanol, dexamethasone, tetracycline and 17-β-estradiol inducible systems have been widely used in science to study gene function (Gatz et al., 1992, 1996; Zuo et al., 2000; Roslan et al., 2001; Samalova et al., 2005). We hypothesize that by using the domain-specific inducible system we would be able to spatially and temporally control *PIN1* expression in *pin1* mutants and analyze PIN1

function in specific domains (Table1) and growth stages, including seedling stage, vegetative stage and reproductive stage. In this project, we determined to use these domain-specific promoters (*MERISTEM LAYER L1 (ATML1), CLAVATA3 (CLV3), FILAMENTOUS (FIL), SCARECROW (SCR), ATHB-8*, *CUP-SHAPED COTYLEDON3 (CUC3)* and *WERWOLF (WER)* (Table 1) with (Figure 3.1) an estrogen inducible XVE cassette to control *PIN1* expression to analyze short-term and long-term impact of *PIN1* expression for *pin1* plant growth and development, respectively (Sessions et a., 1999; Fletcher et al., 1999; Brand et al., 2002; Sawa et al., 1999; Di Larurenzio et al., 1996; Malamy et al., 1997; Kang et al., 2003; Yadav et al., 2014; Vroemen et al., 2003; Hibara et al., 2006; Ryu et al., 2005; Seo et al., 2011; Zuo et al., 2000). The estradiol-inducible XVE transactivation system contains two transcription units. The first unit will be a tissue specific promoter to regulate the XVE expression, which is a fragment of DNA consisting of the DNA-binding domain of bacterial repressor LexA, the transcription activation domain of VP16 and the regulatory region of the human estrogen receptor (ER). The second unit consists of eight copies of the LexA operators fused to a mini 35S promoter, which controls the *PIN1-GFP* expression (Figure 3.1). Upon induction by estradiol, *PIN1-GFP* expression could be monitored by confocal microscope. The estrogen inducible cassette and tissue-specific promoters would allow turning on *PIN1* expression at any developmental stage in specific cell files (Zuo et al., 2000; Siligato et al. 2016). However results from my project suggest that the estrogen inducible system is only good for short-term use to induce gene expression.

3.2 Results

3.2.1 Analysis of PIN1 expression under induction in specific domains

Heterozygous plants from T-DNA insertion line *pin1-7* were used for transformation since the homozygous *pin1* mutants are infertile. To investigate the GFP fused *PIN1* expression, two transgenic lines of T2 generation for each tissue-specific promoter was selected. In the following, experimental data is generally shown from one transgenic line. With the supply of estradiol in the growth media, GFP inflorescence signal demonstrated that *PIN1* expression is inducible in cell-type restricted domains under the control of tissue-specific layer promoters (Table 1). To inspect the spatial pattern of *PIN1* expression in SAM L1 layer, *proATML1::XVE>>PIN1-GFP* transgenic seedlings, in which the *PIN1-GFP* is driven by *ATML1* promoter with 3-kb DNA fragment upstream of the *ATML1* gene start codon, were observed under the confocal after induction. *PIN1-GFP* signal was detected in the shoot epidermal cells, including SAM, cotyledon and hypocotyl (Figure 3.2A, C). Strong signal was also observed at the RAM epidermal cells (Figure 2.2B). Examination of *proCLV3::XVE>>PIN1-GFP* transgenic seedlings under induction found *PIN1-GFP* expressed at the SAM (Figure 3.2D). GFP signal was also detected in the central cells of the root cap (Figure 3.2E).). In the *proCUC3::XVE>>PIN1-GFP* lines supplied with inducers, GFP signal was shown at a narrow area of the shoot apical with a few cells spread onto the petiole epidermis (Figure 3.2F). Induction of the *proFIL::XVE>>PIN1-GFP* transgenic lines showed tissue-specific expression pattern as reported, however weak signal was repeatedly detected in a marrow cell files in the root vascular (Figure 3.2G, H). Under induction, the GFP signal of the *proATHB-8::XVE>>PIN1-GFP* line was observed in the cotyledon vein cells and

epidermis (Figure 3.2I), procambial cells (Figure 3.2J). In the *proSCR::XVE>>PIN1-GFP* lines, the PIN1-GFP signal was detected in the endodermis under induction (Figure 3.2K) (Malany et al., 1998). The induced seedlings of *proWER::XVE>>PIN1-GFP* lines, GFP signal has been detected at the epidermal cells in the root and shoot (Figure 3.2L and M). To our surprise, under induction the *proWER*:*XVE>>PIN1-GFP* transgenic plants grew curly as agravitropic response (Figure 3.3E). The PIN1-GFP signal was examined (Figure 3.3L, M). However, after two more days, the treated transgenic plants (Figure 3B) started growing downwardly as col-0 (Figure 3.3F). PIN1-GFP signal disappear from the PM. It still needs further experiment to find whether PIN1 expression is inhibited or only the protein distribution changed.

3.2.2 The impact of PIN1 leaky expression on transgenic plants growth

Although previous studies from Zuo and colleagues indicated that the XVE is tight, weak GFP signal was repeatedly observed in SAM in multiple promoter lines without the supplement of inducers (Figure 3.4) (Zuo et al., 2000; Siligato et al., 2016). I have carefully examined transgenic seedlings with leakiness at the shoot apical from the *proCUC3::XVE>>PIN1-GFP, proFIL::XVE>>PIN1-GFP* and *proWER::XVE>>PIN1-GFP.* With induction, the GFP signal appeared cell type specificity pattern (as shown in figure 3.2), however without induction weak GFP signal was still detected at the SAM epidermal cells at different developmental stages. In the embryo, at the triangular stage, *GFP* signal was observed at the upper epidermal cell layer restricted to a few cells around the central meristem area (Figure 3.4A, B, and C).

Previous immunological results indicated that *PIN1* expression and its polarity in the epidermis plays an essential role for organ growth and development (Heisler et al., 2005; Huang et al., 2010). Our observations showed resembled results in the leakiness lines *proCLVp::XVE>>PIN1-GFP, proCUC3::XVE>>PIN1-GFP, proFIL::XVE>>PIN1-GFP, proWER::XVE>>PIN1-GFP*. In the seedling stage, *PIN1-GFP* expression started to restrict to a few cells around the central meristem area (Figure 3.5A, C, E and G). Then until the bolting stage, *PIN1-GFP* was expressed in the shoot apical with polarization to the young organs (Figure 3.5B, D, F and H). The leaky expression mainly happened at the SAM L1 layer (Figure 5). Some of the plants showed abnormal rosette leave growth resembled to *pin1* vegetative growth. The inflorescence was able to produce a few siliques, however the inflorescence head would terminate early and grow into a pin-shaped head (Figure 3.6).

3.2.3 Effect of 17-β-estradiol on plant growth

One of the big concerns for inducible systems is the toxicity of the inducers to the plant growth that may abduct the final results. The most used inducers are ethanol, dexamethasone, tetracycline and 17-β-estradiol for different inducible systems (Gatz et al., 1992, 1996; Zuo et al., 2000; Roslan et al., 2001; Samalova et al., 2005). Tetracycline has been reported created ER stress in plants and make the plants appear unhealthily yellowish for long time application. Zuo and his colleagues found 17-β-estradiol is no toxicity to plant growth (Zuo et al., 2000).

For my project, I plan to test the impact of *PIN1* expression at different stages for the *pin1* growth and development including rosette leaf position, leaf serration, flower

development and inflorescence development. Arabidopsis cannot finish a cycle on a plate and it is necessary to test the 17-β-estradiol toxicity in the soil. First, we examined the impact of 17-β-estradiol and 4-hydroxyl tamoxifen treatment on the seedling cotyledon area, hypocotyl and primary length (Figure 7). Under 2μM β-estradiol treatment for one-week, the cotyledon-area, hypocotyl length and primary root growth is no significant difference between control and treated plants. With all the other treatments, the cotyledon-area is significantly smaller than control plants. With 4μM 17-β-estradiol treatment or higher concentration for one week, the hypocotyl length of treated plants is significantly shorter than control. However, there is no significant difference under the concentration from 2μM β-estradiol until 8μM β-estradiol + 8μM 4-HD. Once the β-estradiol concentration is above 2μM, the primary root length is significantly shorter than control plants. However, there is no significant difference under the concentration from 2μm β-estradiol until 4μM β-estradiol + 4μM 4-HD. Overall, 2μM β-estradiol does not significantly affect the plant growth. And the treatment has the least impact on the hypocotyl growth.

3.3 Discussion

The inducible system is a versatile tool to manipulate gene expression, however the selection of non-leaky lines is a very important step for further analysis. Although previous studies have shown that estrogen inducible system is tight and responsive to induction (Zuo et al., 2000; Siligato et al., 2016), it has been difficult to find a line without leaky expression. More than twenty lines for each domain-specific promoter have been analyzed. Since all the layers are originated from the shoot apical meristem region,

in the process of finding non-leaky lines, PIN1-GFP signal has been observed in the SAM L1 layer (Figure 3.4; Figure 3.5). The size of T2 seeds is bigger than wild type and it is especially clear after the seeds soaked. Although most of the lines show correct domain specific expression, the leakiness has hindered further analysis. As a source of aerial organs, the surrounding cells also regulate genes expression from the SAM cells. It has found that leakiness rates also depend on the promoters, which are selected to express the genes of interest (Siligato et al., 2016). Studies of the tetracycline inducible system discovered that increased leakiness happened in the rapidly dividing cells (De Veylder et al. 2000). We postulated that: (1) the mini 35s promoter in the XVE cassette might be active in some regions for unknown reasons; (2) In the construct *PIN1* gene was cloned with 3'-UTR and the UTR might have some regulatory roles in *PIN1* expression.

The SAM contains a central zone consisted of a group of large and slowly dividing cells and a peripheral zone consisted of a group of small and rapidly dividing cells (Vernoux et al., 2010). The cells in the central zone are maintained undifferentiated and replenish the peripheral zone stem cells where initiates the lateral organs (Vernoux et al., 2010). Here we present that two-thirds *pin1* plants rosette leaves are largely normal with leaky *PIN1* expression in the surrounding cells of central zone, but not the inflorescence defects.

In the aim to identify the non-leaky and highly inducible lines, more than twenty lines have been analyzed with 17-β-estradiol induction or without induction for each domain-specific promoter. Observations of seedlings and early bolting stage plants found that the leakiness was mainly happened in the SAM L1 layer (Figure 5). The images of

the embryos further showed that the leakiness is only located in a few cells close to the central zone of the SAM (Figure 3.4). PIN1 function in those cells appears largely complement the leaf phenotypes including abaxial-adaxial polarity, leaf initiation and separation, which is often observed in *pin1* homozygous plants (Figure 3.5B). Without *PIN1* expression in the central region of SAM, the inflorescence terminates with pin-like head with a few siliques (Figure 3.5C, D, E, F). These results suggest that PIN1-mediated auxin flux in the cells of the central zone is critical for the lateral organ development especially flower initiation and development. It still needs further analysis that whether: (1) PIN1-mediated auxin flux is required for plants to increase the SAM size big enough for future flower initiation and growth; (2) PIN1 function in the central zone is necessary for the stem cells of the central region to replenish the peripheral zone stem cells during mature stage; (3) or it affects the inflorescence from both ways.

As a chemical there are concerns about its impact on the plant growth. Results of Zuo and his colleagues suggested that estrogen concentration lower than 5μm does not pose any negative effect on plants (Zuo et al. 2000). Under some condition, 4-hydroxyl tamoxifen is also used together with 17-β-estradiol to enhance the inducibility. Here we tested toxicity of both chemicals, 17-β-estradiol and 4-hydroxyl tamoxifen does not interfere the plant growth including cotyledon expansion, hypocotyl length and primary root elongation with 2μM concentration. Even under 4μM treatment, the chemical does not affect the primary root growth. Siligato et al. has reported that humidity, temperature and light level can affect the toxicity (Siligato et al., 2016). It explains that the toxicity

results might be varied between different laboratory set-ups and indicates the necessity to establish optimal growth condition before using the system.

Examination of 17-β-estradiol on plant growth in the soil was also done with Col-0, *pin1-7* and one transgenic line (*proATHB-8::XVE>>PIN1-GFP*) (Figure S1). Pictures showed plants showed reduced rosette size and smaller rosette leaves with 17-β-estradiol supplement. Recently, Siligato et al showed that 17-β-estradiol has slow diffusibility (Siligato et al., 2016), which may prevent its use on big and mature plants. The estrogen inducible XVE system is a powerful and effective tool for analyzing gene function. However, due to its toxicity, low diffusibility and leakiness, especially the low diffusibility and leakiness, it is difficult to test PIN1 function using this system.

3.4 Material and Methods

Plant Material

SALK_047613 line (*pin1-7*) was used for transformation and analysis. Genotyping of mutants and transgenic lines is as described in chapter 2 (Material and Methods).

Plant Growth Conditions

For seedlings used for confocal imaging, sterilization of T2 seeds was described in chapter 2 (Material and Methods). Seedlings were grown on 0.8% phytoblend plates, containing 1/4 Murashige & Skoog basal salts, 0.5% sucrose, 2 μM 17-β-estradiol, pH 5.5, at 21-23°C, with 16 h of daylight at 100 μmol m^{-2} s^{-1}. For the adult plants used for phenotyping, seeds were sterilized as in chapter 2 (Material and Methods). Sterile seeds were plates on ¼ Murashige & Skoog medium, 0.5% sucrose, 0.8% phytoblend,

10mg/L Basta plates, and then grown under continuous 100 μM $m^{-2}s^{-1}$. 5day seedlings were transferred to soil and grown in the greenhouse under natural light in the summer. Plants were irrigated once per week with acidified water supplemented with a combination of two water-soluble to provide the following (in mg/L): 200 N, 26 P, 163 K, 50 Ca, 20 Mg, 1.0 Fe, 0.5 Mn and Zn, 0.24 Cu and B, and 0.1 Mol. Nitrate form was 76% of nitrogen provided. The rest was irrigated as necessary. Irrigation water was supplemented with 93% sulfuric acid (Brenntag, Reading PA) at 0.08 mL/L to reduce alkalinity to 100 mg/L and pH to a range of 5.8 to 6.2.

Construction of transgenes and Plant Transformation

The single-site Gateway®-compatible pEarleyGate 100 was used as a backbone and the 35s promoter before the gateway cloning cassette in pEarleyGate 100 was removed by PCR to generate pEarleyGate 100Δ (Earley et al., 2006). Domain-specific promoters (Table1) were cloned into pEarleyGate 100Δ by Infusion-PCR (Spiliotis, 2012). XVE cassette was inserted right next to the domain-specific promoters through Infusion-PCR. The entry clone of PIN1-GFP was constructed as described in chapter 3 (material and methods). Finally, PIN1-GFP was inserted right after the XVE cassette through LR reaction (Fig.3.1) or any other gene of interest for further researches (Spiliotis, 2012). Primers are listed in Table 4. Transformation was described in chapter 2 (Material and Methods).

Microscopy

Seedlings were observed under the Zeiss LSM710 confocal microscope to confirm the PIN1 expression. Primary roots and shoot apex were imaged. Microscope set-up is as

describe in chapter 2 (Material and Methods). For the leakiness lines, two-week old plants were grown on the plates were cut off and only the central shoot apical parts were imaged. Florescent signal was collected under different tracks: chlorophyll set-up is 633nm (2.4%) excitation and 674–721 nm emission, GFP setting is 488 nm (20%) excitation and 515–550 nm emission. Images were using Zen 2009 software and Photoshop CC software (Adobe Systems).

Table 4. Primers used to amplify domain-specific promoters and XVE cassette.

Primer	Sequence
XVE-F	TCTCAGAGCCCTCGAGAATGAAAGCGTTAACGGCC
XVE-R	AACTTGTGATCTCGACGACTAGCTTCAGCGTGTCC
XVE-R1	CACCGTACTCGTCAATTCCAA
XVE-F3	TCCTGATGATTGGTCTCGTC
XVE-R3	AGTTAGGTCGAGTCATTTTTG
CLV3-F	TCTCAGAGCCCTCGAGATCATATCCATAAAATTAATCGAA
CLV3-R	CGCTTTCATTCTCGAGAGAGAGAAAGTGACTGAGTG
CUC3-F	TCTCAGAGCCCTCGAGATCCTTACCTTTGCAAGAATT
CUC3-R	CGCTTTCATTCTCGAGCTTTTACTTAATATAACTGAAAAA
proATHB8-F	TCTCAGAGCCCTCGAGACGGATAAACCAATTTTCAAATG
proATHB8-R	CGCTTTCATTCTCGACTTTGATCCTCTCCGATCT
proATML-F	TCTCAGAGCCCTCGAGCATTTACACATCCTGTCGTT
proATML-R	CGCTTTCATTCTCGAGTGGATTCAGGGAGTTTCTTT
pSCR-F	TCTCAGAGCCCTCGAGAACCTCGAGAACCCTCGAGAT
pSCR-R	CGCTTTCATTCTCGAGGGAGATTGAAGG
FIL-F	TCTCAGAGCCCTCGAGATTAAGGAATGACAACAACGG
FIL-R	CGCTTTCATTCTCGAGCTTTTTTGTAAGAAGGGGAAA
WER-F	TCTCAGAGCCCTCGAGATGAAAGGCGGGCCCAATA
WER-R	CGCTTTCATTCTCGAGTCTTTTTGTTTCTTTGAATGATA

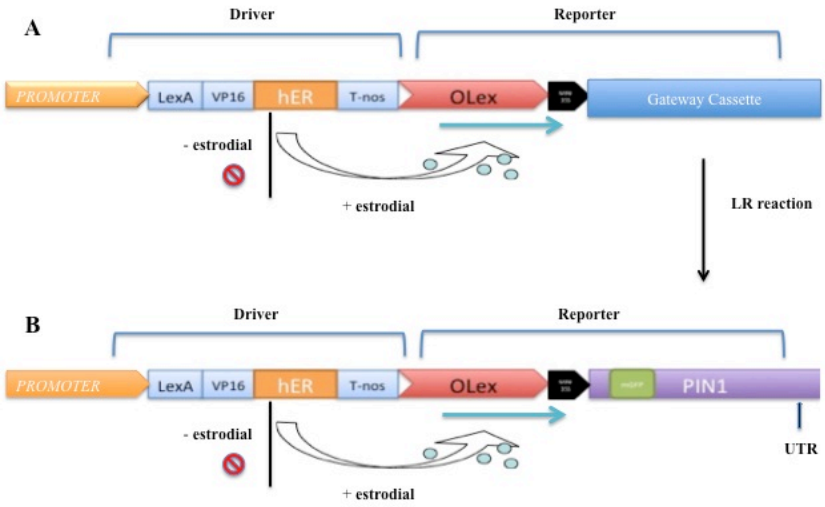


Figure 3.1. **Schematic diagram of the estrogen receptor-based inducible system with basta resistance selectable marker.**

A, Promoter sequence and XVE cassette were cloned into pEarleyGate100 before the Gateway cloning site by In-Fusion PCR to generate a pEarleyGate100Δ. B, *PIN1-GFP* was cloned into an entry vector. Then *PIN1-GFP* was recombined into to generate a pEarleyGate100Δ through LR reaction to generate a destination vector. The estradiol-inducible XVE transactivation system contains two transcription units. The first unit will be a tissue specific promoter to regulate the XVE expression, which is a fragment of DNA consisting of the DNA-binding domain of bacterial repressor LexA, the transcription activation domain of VP16 and the regulatory region of the human estrogen receptor (ER). The second unit consists of eight copies of the LexA operators fused to a mini 35S promoter, which controls *PIN1-GFP* expression. Circles represent estradiol, which binds and activates the tissue specific expressed ER, in turn results in the transcription of *PIN1*. (Zuo et al., 2000).

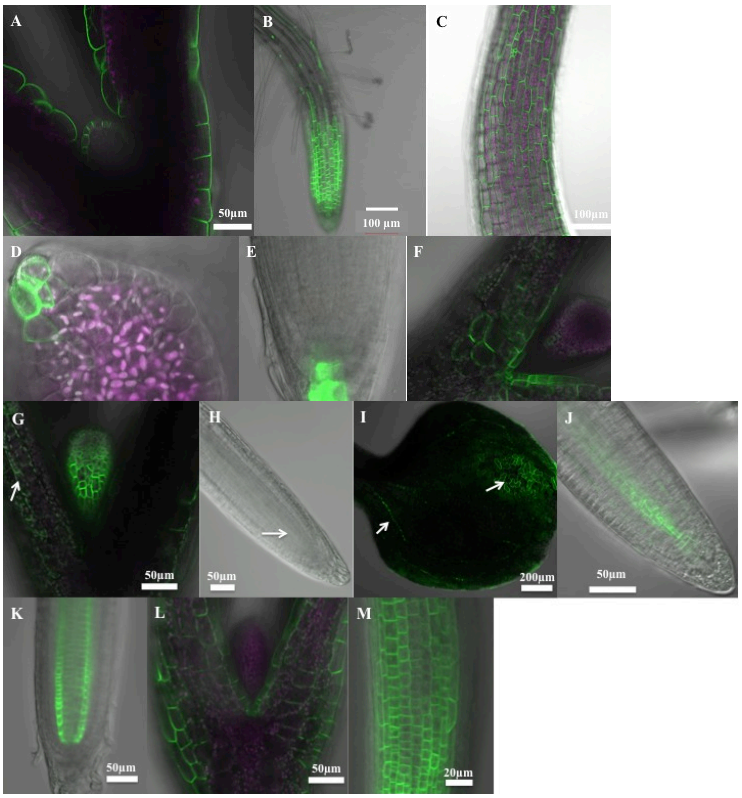


Figure 3.2. Visualization of *PIN1-GFP* in *Pro*:*XVE>>PIN1-GFP* transgenic plants under 17-β-estradiol induction.

A-C, *proATML1::XVE>>PIN1-GFP*. D-E, *proCLV3::XVE>>PIN1-GFP*. F, *proCUC3::XVE>>PIN1-GFP*. G-H, *proFIL::XVE>>PIN1-GFP*. I-J, *proATHB-8::XVE>>PIN1-GFP*. K, *proSCR::XVE>>PIN1-GFP*. L-M, *proWER::XVE>>PIN1-GFP*. Scale bar is as indicated. Violet color shows chlorophyll autofluoresence in all the images. Arrows point to the expression area. T2 seeds were used to image. 4-day-old seedlings were imaged by zeiss LSM710 confocal microscope.

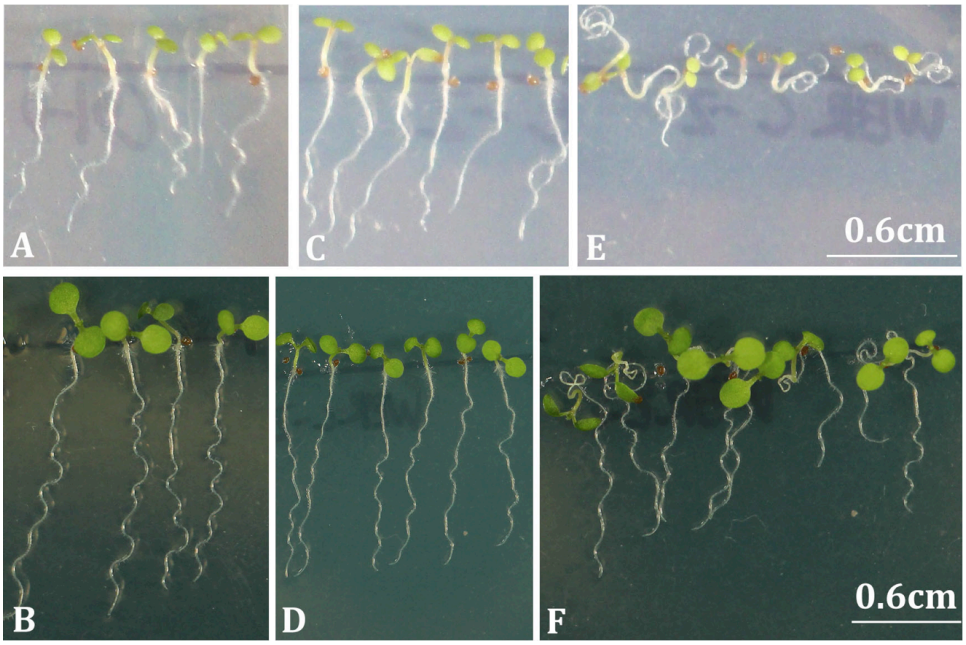


Figure 3.3. PIN1 instability on the PM of root epidermal cells under induction.

proWER:*XVE>>PIN1-GFP* transgenic plants show agravitropic response when treated with with 2μM 17-β-estradiol for three days (E). And the agravitrpoic phenotype disappeared after two-more-days treatment (F). A and B, col-0. C and D, Plants *proWER*:*XVE>>PIN1-GFP* transgenic line without treatment grow as the col-0. E, *proWER*:*XVE>>PIN1-GFP* Plants were grown on the ¼ Murashige & Skoog medium with 0.5% sucrose, 2μM 17-β-estradiol, pH5.5 in the growth chamber under 100μM $m^{-2}s^{-1}$ light . After 3 days seedlings showed curled hypocotyl and root and F, plants start growing normal after five days growth with inducers. Bar is as indicated.

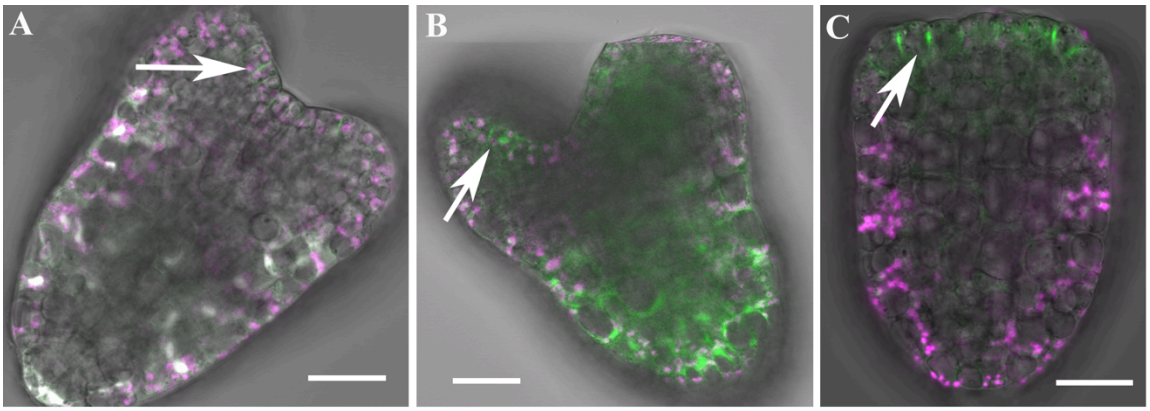


Figure 3.4. Visualization of *PIN1-GFP* in the embryos of *Pro*:*XVE>>PIN1-GFP* transgenic plants without 17-β-estradiol induction.

A, *proCUC3::XVE>>PIN1-GFP.* B, *proFIL::XVE>>PIN1-GFP.* C, *proWER::XVE>>PIN1-GFP.* Scale bar equals to 20μm. Violet color shows chlorophyll autofluoresence in all the images. Arrows point to the expression cells. Plants were grown in the green house under natural light in the summer. After six weeks, siliques were removed and peeled under the microscope. The embryos were imaged with zeiss LSM 710 confocal microscope. T2 transgenic seeds were grown in the soil for three weeks until and then embryos were peeled and imaged by zeiss LSM710.

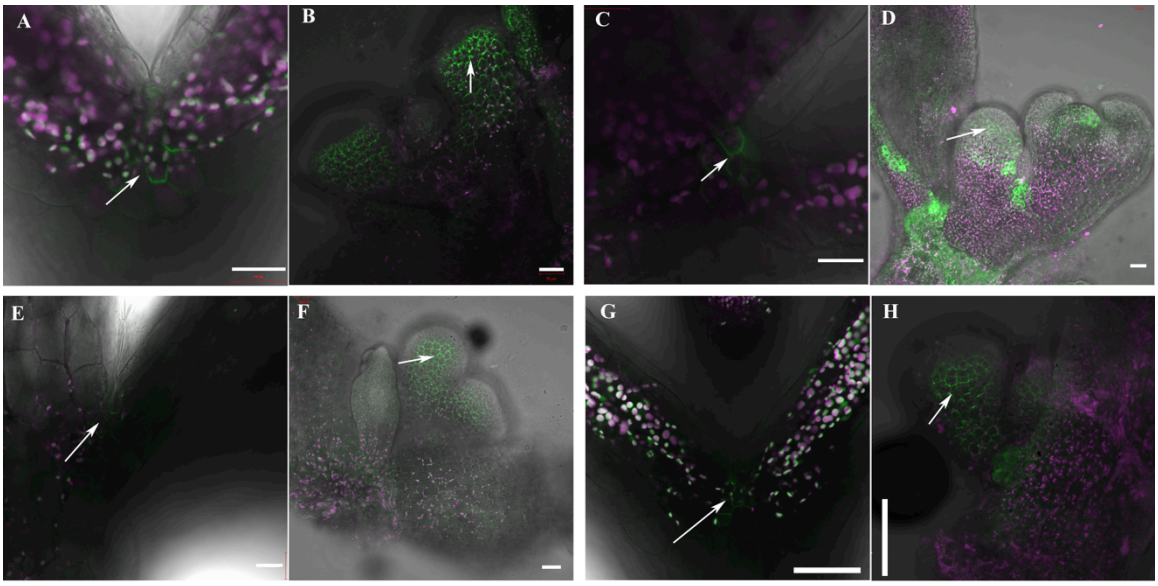


Figure 3.5. Example of PIN1-GFP signal and polarity in seedlings and inflorescence meristem of some *Pro*:*XVE>>PIN1-GFP* transgenic lines without induction.

A, *PIN1-GFP* in *proCLV3::XVE>>PIN1-GFP* seedlings and arrow points to the leaky region.
B, *PIN1-GFP* expression in *proCLV3::XVE>>PIN1-GFP* inflorescence meristem and arrow shows *PIN1-GFP* polarized to the young developing organ.
C, PIN1-GFP in *proCUC3::XVE>>PIN1-GFP* seedlings and arrow points to the cells have *PIN1-GFP* expression.
D, *PIN1-GFP* expression in *proCUC3::XVE>>PIN1-GFP* inflorescence meristem and PIN1-GFP polarizes to the incipient flower.
E, PIN1-GFP in *proFIL::XVE>>PIN1-GFP* seedling and arrow shows the leaky signal.
F, *PIN1-GFP* expression in *proFIL::XVE>>PIN1-GFP* inflorescence meristem and arrow indicates PIN1-GFP polarized to the young flower buds.
G, PIN1-GFP in *proWER::XVE>>PIN1-GFP* seedling (arrow).
H, *PIN1-GFP* expression and polarity in *proWER::XVE>>PIN1-GFP* inflorescence meristem(arrows).
Scale bar is 20µm. Violet color shows chlorophyll autofluoresence in all the images. Plants were grown on the ¼ Murashige & Skoog medium with 0.5% sucrose, pH5.5 in the growth chamber under 100µM $m^{-2}s^{-1}$ light. After 4 days seedlings were imaged with zeiss LSM 710 confocal microscope. Then two-week-old SAM of the early bolting stage was dissected under the stereoscope. Bar is as indicated.

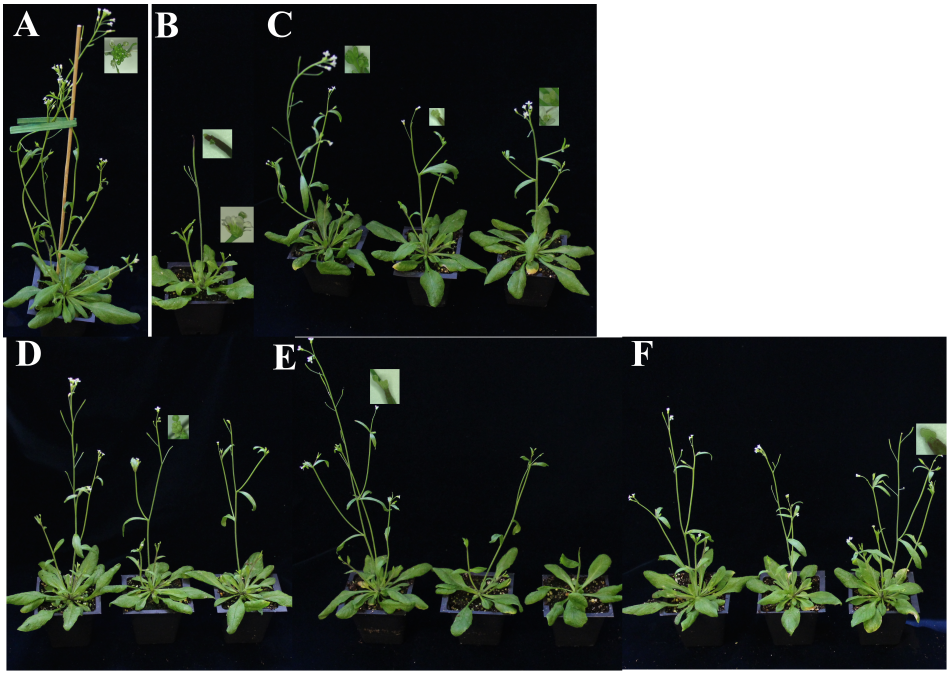
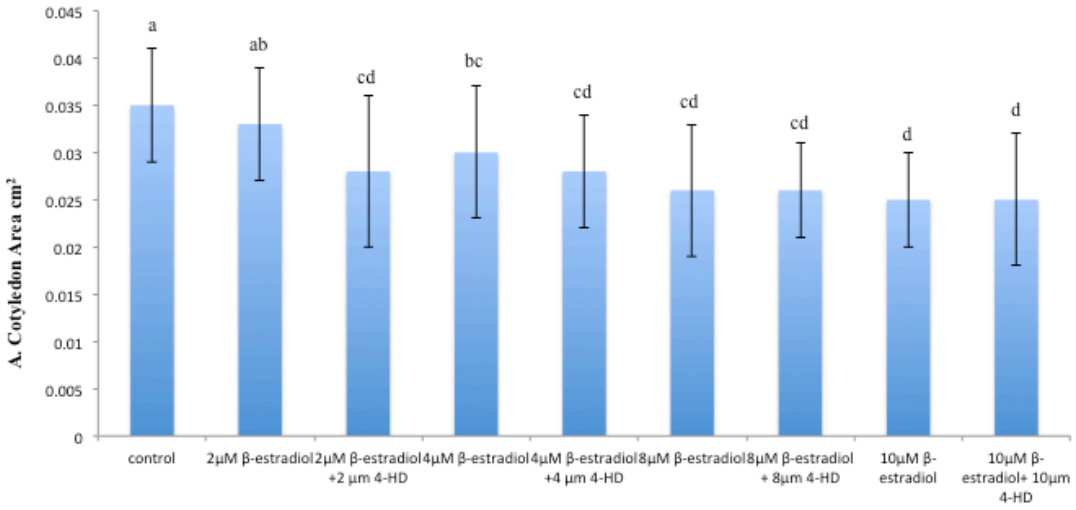
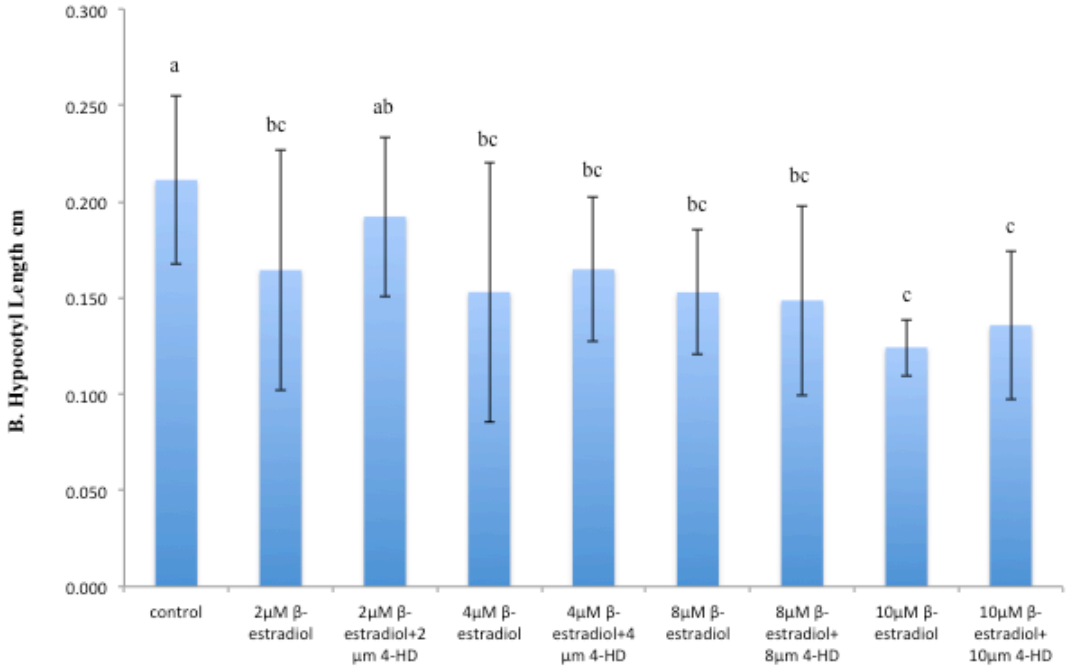


Figure 3.6. Plant growth phenotypes of col-0, *pin1* and leakiness lines.

A. Col-0 and the primary inflorescence head.
B. Homozygous *pin1* plants have pin-shaped inflorescence head and abnormal flower.
C. *proCLV3::XVE>>PIN1-GFP,* D. *proCUC3::XVE>>PIN1-GFP,* E. *proFIL::XVE>>PIN1-GFP,* F. *proWER::XVE>>PIN1-GFP* transgenic plants with leakiness have largely normal rosette, some siliques and pin-like inflorescence head. Seeds were grown on the ¼ Murashige & Skoog medium with 0.5% sucrose, pH5.5 in the growth chamber under 100μM $m^{-2}s^{-1}$ light for 4 days. Then 4-day-old seedlings were transferred into soil in the green house under natural light in the summer.


A. Cotyledon Area cm²
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
a
ab
cd
bc
cd
cd
cd
d
d
control
2μM β-estradiol
2μM β-estradiol +2 μm 4-HD
4μM β-estradiol
4μM β-estradiol +4 μm 4-HD
8μM β-estradiol
8μM β-estradiol + 8μm 4-HD
10μM β-estradiol
10μM β-estradiol+ 10μm 4-HD


B. Hypocotyl Length cm
0.300
0.250
0.200
0.150
0.100
0.050
0.000
a
bc
ab
bc
bc
bc
bc
c
c
control
2μM β-estradiol
2μM β-estradiol+2 μm 4-HD
4μM β-estradiol
4μM β-estradiol+4 μm 4-HD
8μM β-estradiol
8μM β-estradiol+ 8μm 4-HD
10μM β-estradiol
10μM β-estradiol+ 10μm 4-HD

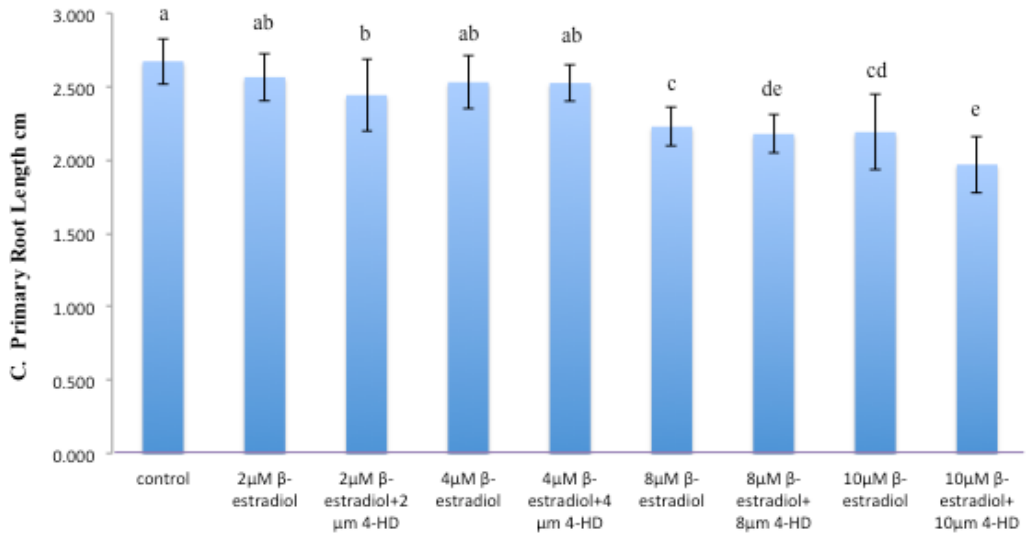


Figure 3.7. Effect of 17-β-estradiol and 4-hydroxyl tamoxifen on *Arabidopsis* seedlings growth.

A. Cotyledon area, B. Hypocotyl length and C. Primary root lengths were measured 7 days after germination. The average length for each tissue was calculated from 8 seedlings. Measurement was done in imageJ. Values represent means. Error bars represent standard deviation (ANOVA, $p<0.05$, Fisher's LSD). For (A), (B) and (C), bars without sharing the same letter are significantly different. At 2µM 17-β-estradiol, the cotyledon and hypocotyl growth is similar as control. When 17-β-estradiol concentration at 2µM and 4µM, the primary root length resembles to control plants. *Arabidopsis* seedlings were grown on the ¼ strength of Murashige & Skoog medium plates supplemented with 0.5% sucrose, 17-β-estradiol and 4-hydroxyl tamoxifen under 16h light 100µM $m^{-2}s^{-1}$ for 7 days. Equal concentration of DMSO was added in the media before making the control plates.

Supplemental material

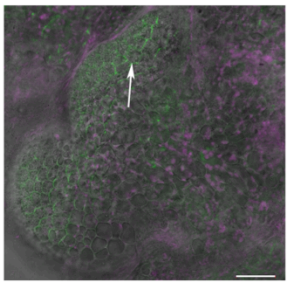


Figure S1. *PIN1-GFP* expression and polarity in *proATHB-8::XVE>>PIN1-GFP* inflorescence meristem(arrows).

Scale bar is 20μM. Violet color shows chlorophyll autofluoresence in the image. Seeds were surface sterilized with 10% bleach for 10 minutes and rinsed 5 times with autoclaved water. Then seeds were grown on the ¼ Murashige & Skoog medium supplemented with 0.5% sugar for two weeks. Seedlings were imaged by zeiss LSM 710.

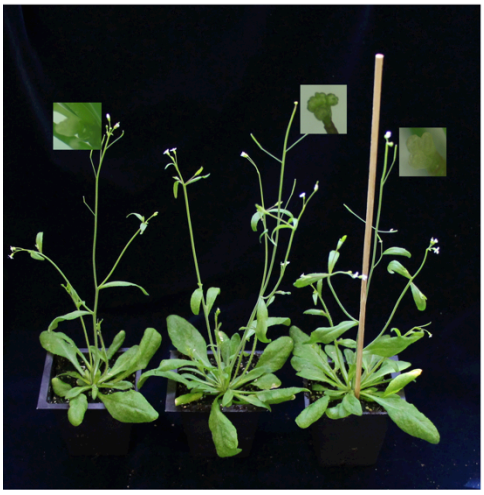


Figure S2. ***proATHB-8::XVE>>PIN1-GFP*** **transgenic plants with leakiness have largely normal rosette leave, secondary inflorescence, some siliques and pin-like inflorescence head.**

Plants were grown in the greenhouse under natural light in the summer.

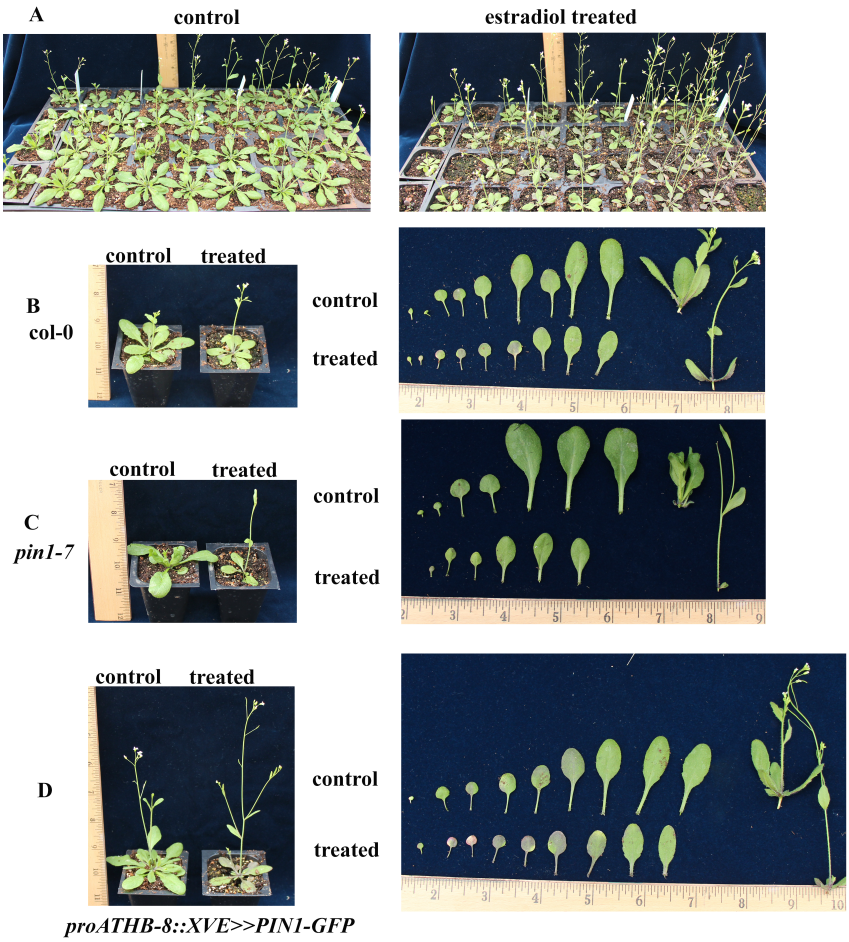


Figure S3. Effect of 17-β-estradiol on Arabidopsis seedlings growth in the soil.

5-day-old Arabidopsis seedlings were transferred into soil and then 1μM 17-β-estradiol was added for each watering. After three weeks, plants were examined. A. The whole tray growth status. B. Col-0 plants and single rosette leaf. C. *pin1-7* plants and single rosette leaf. D. *proATHB-8::XVE>>PIN1-GFP* plants and single rosette leaf. Bar is as shown. Plants were grown in the greenhouse in the summer. Plants were irrigated once a week with acidified water supplemented with miracle grows fertilizer. The other times were irrigated as necessary with water contained 93% sulfuric acid (Brenntag, Reading PA) at 0.08 mL/L to reduce alkalinity to 100 mg/L and pH to a range of 5.8 to 6.2.

LIST OF REFERENCES

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